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14. ABSTRACT

Our hypothesis is that PEITC is an effective preventive agent in breast cancer. Although a number of mechanisms may be involved, one mechanism that has not been explored is the potential for PEITC to alter the synthesis and elimination of estrogens. Our specific aims are: (1) to evaluate the efficacy of dietary PEITC, at relevant doses, in preventing or delaying the onset of breast cancer in estrogen-dependent breast cancer animal models, (2) to evaluate the effects of PEITC on 17 β -estradiol (E_2) and metabolite concentrations in plasma and tumor samples, and (3) to determine changes in enzyme activity and/or mRNA/protein expression in tumor and liver samples, for metabolic pathways which may be altered based on the E_2 and E_2 metabolite profiles. Our studies have characterized the pharmacokinetics of PEITC in rats, developed a LC/MS/MS assay for 17 β -estradiol, and demonstrated in preliminary studies that PEITC can alter estrogen metabolism in human mammary and breast cancer cells and in rat liver. Additionally, we found that low concentrations of PEITC can alter the expression of some estrogen-responsive genes important in breast cancer. These studies provide new findings regarding the chemopreventive effects of the dietary compound PEITC in estrogen-dependent breast cancer.

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P.I.: Marilyn E. Morris, Ph.D.

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INTRODUCTION

Although more than 20 natural and synthetic organic isothiocyanates (ITCs) have been shown to block carcinogenesis, phenethyl isothiocyanate (PEITC), present in cruciferous vegetables, is one of the most extensively studied ITCs because of its high potency against a variety of tumors and its low *in vivo* toxicity. Our hypothesis is that PEITC is an effective preventive agent in breast cancer. Although a number of mechanisms may be involved, one mechanism of PEITC effects that has not been explored is the potential for PEITC to alter the synthesis and elimination of estrogens. Such an effect might be anticipated since PEITC can inhibit Phase I enzymes and induce Phase II enzymes. Our specific aims are: (1) to evaluate the efficacy of dietary PEITC, at relevant doses, in preventing or delaying the onset of breast cancer in estrogen-dependent breast cancer animal models, (2) to evaluate the effects of PEITC on 17beta-estradiol (E₂) and metabolite (E₂ sulfate, E₂ glucuronide, estrone, 2-hydroxyE₂, 4-hydroxyE₂, 2-methoxyE₂, and 4-methoxyE₂) concentrations in plasma and tumor samples, and (3) to determine changes in enzyme activity and/or mRNA/protein expression in tumor and liver samples, for metabolic pathways which may be altered based on the E₂ and E₂ metabolite profiles. Studies will evaluate the effects of PEITC in two estrogen-dependent animal breast cancer models (1) N-methyl-N-nitrosurea (NMU)-induced carcinogenesis in rats, and (2) estrogen-induced mammary gland cancer in ACI rats. These studies will provide new findings regarding the chemopreventive effects of the dietary compound PEITC in estrogen-dependent breast cancer.

BODY

Statement of Work

Task 1. To determine the dose of PEITC to be used in diets.

- a. Perform preliminary pharmacokinetic studies in rats to obtain bioavailability and clearance data.

- b. Have diets prepared with and without PEITC. Two diets will be prepared containing PEITC that should produce average steady state plasma concentrations of 1 μM and 10 μM .

This task has been completed, and the diets have been prepared based on the pharmacokinetic parameters for PEITC determined in pharmacokinetics and bioavailability determined in rats. Performing pharmacokinetic studies in rats were crucial for this Task.

Bioavailability and Pharmacokinetics of PEITC in Rats (Abstract published AAPS J. 6(4):Abstract T3339, 2004; manuscript accepted for publication in Pharm. Res.)

Purpose. Our objective was to examine dose-dependent pharmacokinetics and oral bioavailability of unchanged PEITC, as well as its pH- and temperature-dependent stability and protein binding

Methods: Male Sprague-Dawley rats were administered PEITC at doses of 2, 10, 100 or 400 $\mu\text{mol/kg}$ i.v. or 10, 100 or 400 $\mu\text{mol/kg}$ orally. PEITC was prepared in 15% hydroxy-propyl- β -cyclodextrin. Plasma samples were collected at 5, 15, 30 min and 1, 2, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h and analyzed by a LC/MS/MS assay. Pharmacokinetic data were analyzed by WinNonlin for non-compartmental analysis and ADAPT II for compartmental analysis.

Results: The mean plasma concentrations (\pm SD) of PEITC after iv administration (Fig. 1) and oral administration (Fig. 2) are given below. With an increase in the PEITC dose, elimination half-life ($t_{1/2}$) and time to C_{max} (t_{max}) increased, maximal plasma concentrations (C_{max}) increased but not proportionally, and oral bioavailability (F) decreased. At the highest dose, CI was decreased while V was increased (Table 1). The plasma concentration profile of PEITC after i.v. administration can be well characterized by a three-compartment model with Michaelis-Menten elimination and distribution.

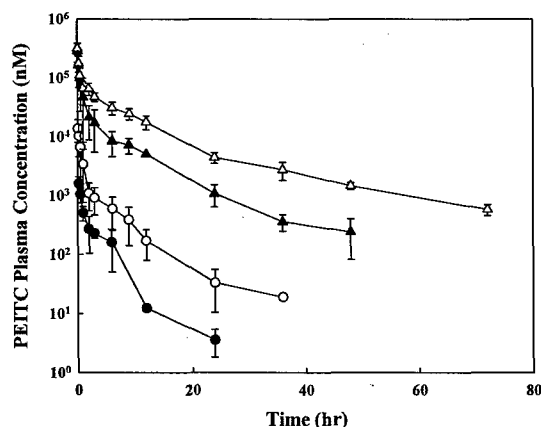


Fig. 1. The plasma concentration profile of PEITC after intravenous administration. Rats were intravenously dosed with 2 (\bullet), 10 (\circ), 100 (\blacktriangle) or 400 $\mu\text{mol/kg}$ (\triangle) of PEITC. Data are expressed as mean \pm SD, $n = 3$ or 4.

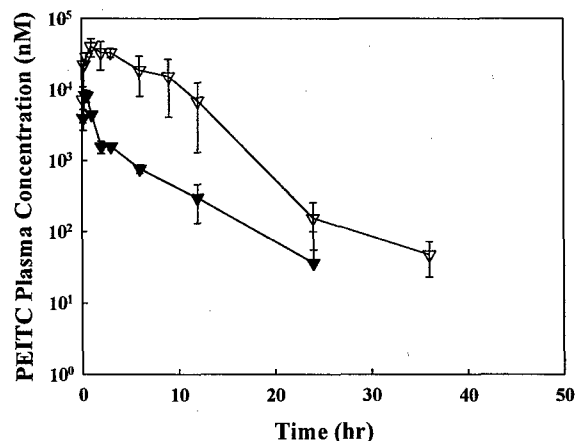


Fig. 2. The plasma concentration profile of PEITC after oral administration. Rats were intravenously dosed with 10(\blacktriangledown) and 100 $\mu\text{mol/kg}$ (\triangledown) of PEITC. Data are expressed as mean \pm SD, $n = 3$ or 4.

Table 1. The pharmacokinetic parameters of PEITC in rats after oral administration. Noncompartmental analysis was performed by WinNonlin.

Abbreviations for the parameters: C_{max} , maximal plasma concentration; t_{max} , time to reach C_{max} ; F, bioavailability.

Dose ($\mu\text{mol/kg}$)	10	100
AUC ($\mu\text{M}\cdot\text{h}$)	19.89 ± 3.27	298.7 ± 139.4
t_{max} (h)	0.44 ± 0.10	2.0 ± 1.0
C_{max} (μM)	9.2 ± 0.6	42.1 ± 11.4
F (%)	115	93

Conclusions: PEITC is a dietary component with high oral bioavailability and low clearance in rats. Nonlinear elimination and distribution is evident at high doses. In contrast to many dietary components, PEITC has excellent bioavailability.

Task 2. To set up assays for 17β -estradiol (E_2), estrone, 2-OHE₂, 4-OHE₂, 2-methoxyE₂ and 4-methoxyE₂. (With consultant)

- Set up GC/MS assay for plasma samples.
- Set up GC/MS assay for tumor tissue and mammary gland samples.
- Validate conditions for hydrolysis of sulfate and glucuronide conjugates of E₂.

Our initial goal was to use a published GC/MS assay to measure E₂ and its metabolites. Our consultant, Dr. James Olson, had previously used this assay in studies with microsomal preparations of liver and lung isolated from rats. However, the sensitivity is low, and exogenous E₂ needs to be added to microsomal preparations. In order to detect physiological concentrations of E₂, we have modified and validated a human radioimmunoassay (Diagnostic Products, Inc.), and can detect plasma, liver and mammary gland E₂ concentrations.

LC/MS/MS Assay for estradiol and metabolites

To develop and validate a liquid chromatography-tandem mass spectrometry (LC/MS/MS) assay for the measurement of physiological concentrations of 17β -estradiol and its metabolites.

Methods: Our LC/MS/MS system consists of a PE SCIEX API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a heated nebulizer interface, a series 2000 Perkin-Elmer pump, and a series 2000 Perkin-Elmer autosampler (Shelton, CT). We have used Oasis HLB SPE cartridges for sample clean-up. Plasma samples (1 ml) are applied to the column and eluted with methanol. The methanol is evaporated and the sample reconstituted with mobile phase (70/30 methanol/20 mM ammonium hydroxide). E₁, E₂ and metabolites are separated using a phenyl Xterra column, and injection volumes of 10-40 μl were used. So far, we have optimized parameters for E₁, E₂, 2-hydroxyE₂ and 4-methoxyE₂.

Table 2. LC/MS/MS Assay for Estradiol and Metabolites

Compound	Molecular Weight	Fragment Monitored in MRM	Retention time	Lowest concentration detected in mobile phase (with 40uL injection)	Lowest concentration detected in plasma (with 20ul injection)
Estradiol	272.38	145.1	2.89	50pg/ml	500 pg/ml

Estrone	270.37	145.1	3.30	50pg/ml	100pg/ml
2-hydroxyestradiol	288.4	147.2	1.96	1ng/ml	>1ng/ml (?)
4-methoxyestradiol	302.4	286.1	3.08	100pg/ml	100pg/ml
Ethynyl estradiol (internal standard)	296.5	145.1	2.93	50pg/ml	-

A PE SCIEX API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystem, Foster City, CA) equipped with a TurboIonSpray(TIS) interface, a series 2000 Perkin-Elmer pump, a series 2000 Perkin-Elmer autosampler (Shelton, CT) and Analyst 1.3 software is used for data acquisition and processing. The mass spectroscopy is performed in a negative mode using multiple reaction monitoring. Optimal operating parameters of TIS were obtained, with regard to maximum signal intensity of molecular ions and fragment ions, by consecutive infusion of standard solutions of estradiol and its metabolites, using a Harvard syringe pump. The optimum conditions of the interface are as follows: ion spray voltage of -4500 V, pressure of collision gas (N₂) of 2.8mTorr, flow rate of the nebulizer gas (air) and curtain gas (N₂) of 1.2 and 0.8 L/min, respectively. The interface temperature is set at 350°C. The optimal declustering, focusing and entrance potentials are given below. The nebulizer current is 12 µA and fragmentation is induced with a collision energy of -30eV.

The second approach that we have used is to incorporate a derivatization step. Derivatization with dansyl chloride has been used to measure endogenous levels of estradiol and estrone in human plasma. The reaction worked for estrogen and estradiol, but does not work for metabolites possibly due to hindrance from the substituted groups on 2 and 4 position. Optimization of parameters for E₁ and E₂ was performed and levels as low as 100 pg/ml can be detected. The derivatization procedure involves incubation of the sample with 150 µL dansyl chloride in acetone and 50µL sodium carbonate buffer (pH=11) at 60°C for 3 minutes. This is followed by centrifugation and extraction with 1 ml hexane. The hexane is evaporated under a nitrogen stream and the dried samples reconstituted with acetonitrile. The mobile phase was water/acetonitrile (20/80) with 1% formic acid in both phases.

Conclusions: We have 2 LC/MS/MS assays for 17β-estradiol with adequate sensitivity to analyze physiological concentrations. We have also modified a RIA that can be used to measure physiological estradiol concentrations in rats. We have had some success with developing an assay for hydroxylated and methoxylated metabolites by LC/MS/MS, but are able to measure higher concentrations of these metabolites using GC/MS. Conjugated metabolites can be determined after hydrolysis with β-glucuronidase and sulfatase.

- Task 3.* To examine the effect of PEITC in rats with NMU-induced mammary gland tumors.
- Three groups of rats (12-15 per group) will be treated with diets containing either no PEITC, low PEITC or high PEITC. Animals will be assessed weekly for the presence and size of mammary tumors. Latency to tumor development will be determined.
 - After about 120 days, rats are sacrificed and blood, tumor and tissues (lung, lymph nodes and liver) obtained for the evaluation of tumor number and size evaluation, and presence of metastases.

- c. Determine PEITC by LC/MS/MS in plasma and tissue samples.
- d. Analyze plasma and tumor samples for 17β -estradiol (E_2), E_2 following hydrolysis with sulfatase and β -glucuronidase, estrone, 2-OHE₂, 4-OHE₂, 2-methoxyE₂ and 4-methoxyE₂ using GC/MS.

a) Effect of PEITC on rats with NMU-induced mammary gland tumors.

These studies are currently being performed, and animals have been treated with NMU. Sprague Dawley female rats, 43-50 days old were obtained from Harlan Sprague Dawley Laboratories. The diet was obtained from Nutritional Diets and consists of AIN93G diet (using corn oil instead of soy oil) with or without 6 μ mol/gm PEITC (formulated in powder form, stored in vacuum sealed packets of 2.5 kg, to avoid loss of PEITC). Animals were divided into four groups and received Control Diet + NMU; Control Diet + Saline; PEITC Diet + Saline; PEITC Diet + NMU.

The animals received the modified AIN93G diet with or without PEITC (according to group) for 10 days. After 10 days, NMU was injected i.p at a dose of 50mg/kg in animals of group 1 and 4, the second dose was administered 10 days later.

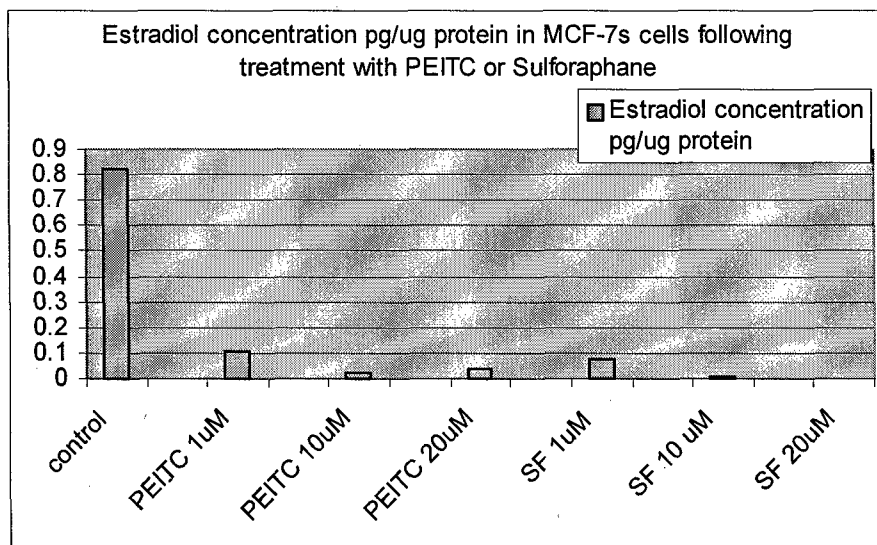
Animals will be evaluated weekly for mammary tumors 2 weeks from administration of the second NMU dose. Evaluation will be done by measuring the tumor size using calipers. At the end of 12 weeks various tissues of the animals will be collected (breast, liver, blood) and evaluated for levels of estradiol and its metabolites (this is in progress).

We have performed preliminary studies, examining the effects of PEITC administration on estradiol liver concentrations, and the metabolism of estradiol in rat liver microsomes (described after Task 5).

b) Effect of PEITC Treatment on 17β -estradiol concentrations in human breast cancer MCF-7 cells.

Our purpose was to determine the effects of PEITC treatment in human breast cancer MCF-7 cells. Cells were cultured and PEITC, sulforaphane (SF) or the vehicle DMSO was added to medium (RPMI 1640 containing 10% fetal bovine serum (FBS), 2mM L-glutamine, penicillin (10 units/ml) and streptomycin (10 μ g/ml)) for 6 hours. The cells were washed in ice-cold phosphate buffered saline (PBS) and collected using cell scraping in PBS, followed by sonication for 5 minutes to lyse the cells before analysis.

Fig. 3



17 β -estradiol concentration were determined using a modified RIA (Diagnostic Products). Protein concentration was determined by the bicinchoninic acid (BCA) assay using a commercially available assay kit (Pierce Biotechnology, Inc., Rockford, IL) and protein concentrations were unchanged with treatment. The results in Figure 6 represent the mean of two samples. Pronounced decreases in 17 β -estradiol concentrations were seen in this study following treatment with PEITC and sulforaphane at 1 μ M concentrations. Further studies are needed to confirm these results.

Conclusion: Decreased concentrations of 17 β -estradiol were observed in cell culture experiments with human breast cancer cells following treatment with PEITC.

c) Effect of oral PEITC treatment on liver estradiol concentrations in rats.

Preliminary studies were conducted in female SpragueDawley rats fed 400 μ mol/kg PEITC (in 15% hydroxypropyl-beta-cyclodextrin) twice daily for 5 days by oral gavage. Animals were sacrificed and blood and liver removed for estradiol analysis by a modified RIA (Diagnostic Products). Physiological concentrations of 17 β -estradiol were decreased in livers of treated animals (Fig. 4), and the rate of estradiol metabolism was increased in liver microsomes of rats treated with oral PEITC (Table 3).

Conclusion: These findings support our hypothesis that PEITC can alter the metabolism of estrogens.

Fig. 4

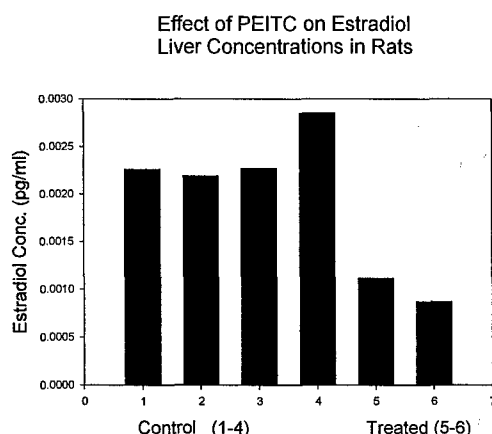


Table 3. Rate of metabolism of 17 β -estradiol in rat liver microsomes. Significantly different, n=4, p<0.005

	Control mean \pm SD	Treated with PEITC mean \pm SD
Rate of metabolism (pmol/mg/min)	94.6 \pm 2.3	128 \pm 7.0

Task 4. To examine the effect of PEITC in ACI rats.

- Three groups of rats (12-15 per group) will be treated with diets containing either no PEITC, low PEITC or high PEITC. Animals will be assessed weekly for the presence and size of mammary tumors. Latency to tumor development will be determined.
- After about 180 days, rats are sacrificed and blood, tumor and tissues (lung, lymph nodes and liver) obtained for the evaluation of tumor number and size and presence of metastases.
- Determine PEITC by LC/MS/MS in plasma and tissue samples
- Analyze plasma and tumor samples for 17 β -estradiol (E₂), E₂ following hydrolysis with sulfatase and β -glucuronidase, estrone, 2-OHE₂, 4-OHE₂, 2-methoxyE₂ and 4-methoxyE₂ using GC/MS.

These studies have not been performed due to a lack of funding. The LC/MS/MS assay for PEITC has been developed, validated and published. The LC/MS/MS assay for E2 and its metabolites is being developed.

Task 5. To determine changes in enzyme activity, mRNA and protein expression for E₂ metabolic pathways which may be altered, based on the E₂ and E₂ metabolite profile.

- a. The enzymes of interest include: aromatase, cytochrome P450(CYP)1A1/2, CYP1B, and various conjugating enzymes (sulfotransferase, UDP-glucuronyltransferase, catechol O-methyltransferase). Sulfatase and β -glucuronidase will also be examined. Activity levels will be determined in tumor and/or liver, using standard assays.
- b. mRNA levels will be determined using a specific metabolic cDNA array from Superarray, so that many enzymes may be screened.
- c. Protein expression will be examined using specific antibodies and Western analysis.

This task has been completed. We have examined the effects of low PEITC concentrations in 2 different cDNA arrays available from Superarray Inc.: 1) Metabolizing Enzymes and 2) Breast cancer genes.

a) Effects of PEITC (3 μ M) treatment on Drug Metabolizing Enzymes in Human Hepatocytes. We chose to determine the potential effect of PEITC on metabolizing enzymes by evaluating the effect of a 5-day incubation with PEITC (3 μ M) on the gene expression in human hepatocytes (In Vitro Technologies). This technique allowed us to examine a wide range of enzymes. We used the GEMArray[®] Q series Human Drug Metabolism Superarrays containing 96 genes encoding for drug metabolizing enzymes. Briefly, cDNA probes were synthesized by reverse transcription using 1 μ g of the control (vehicle treatment) or treated RNA samples as the templates and labeled with biotin-16-dUTP (Roche). The cDNA probes are then denatured and hybridized with GEMArray[®] membranes. The hybridization signal was detected with Kodak Image Station, and the relative abundance of a particular transcript was normalized against the signal of glyceraldehyde 3 phosphate dehydrogenase (GAPDH). The differences between the control and treated RNA samples were evaluated by significant analysis of microarrays (SAM) and student's t-test. There were no significant differences detected. This may reflect the variability observed with the human hepatocyte preparations.

Conclusions: At a concentration of 3 μ M of PEITC, we did not observe any significant changes in mRNA expression of drug metabolizing enzymes. It is likely that any effects of PEITC on enzymes may represent direct effects.

b) Effects of PEITC Treatment on estrogen receptor signaling in human mammary epithelial cells (HMEC) and human breast cancer MCF-7 cells

Methods: HMEC cells were obtained from Dr. Martha Stampfer (Lawrence Berkeley National Laboratory) and were incubated until 60-80% confluence at 1% CO₂ in a 37°C incubator. MCF-7 cells were incubated until 60-80% confluence at 5% CO₂ in a 37°C incubator. HMEC and MCF-7 cells were treated with DMSO (control) or PEITC at one of two concentrations (0.3 or 3.0 μ M) for 48hrs. One of two methods was used to harvest cells from 75ml flask for RNA isolation. Cells were harvested by adding ice-cold 1X PBS and scraping or by using a lysis buffer (Promega SV Total RNA Isolation Kit). Total RNA was isolated from both cell lines by using the SV RNA Isolation System (Promega, Madison WI). RNA was determined

spectrophotometrically at 260 nm. (0.5mg/ml- 5mg/ml are required for the gene array). GEArray Q series Breast Cancer and Estrogen Receptor Signaling Gene Array kit (SuperArray Inc., Bethesda, MD) was used, according to the manufacturer's instructions. cDNA was prepared from total RNA by reverse transcription with MMLV reverse transcriptase (Invitrogen, Buffalo, NY). The cDNA array contains ninety-six genes important in breast cancer and estrogen receptor signaling. Spots were detected using chemiluminescence-labeled biotin (Invitrogen, Buffalo, NY), then hybridized under precisely specified conditions to a positively charged nylon membrane containing the arrayed DNA. After washing, the relative expression level of each gene was analyzed using a Kodak Image Station 440CF.

Data was analyzed using ROI analysis which measures the mean intensity for each gene spot. The background (PUC18/Blank) was subtracted from the spot net mean intensity. The housekeeping genes, β -actin and glyceraldehyde 3 phosphate dehydrogenase (GAPDH), as well as the average of total intensity, were used for normalization. Statistical analysis used the program SAM (Significance Analysis of Microarrays), which is a microarray data analysis package used to correct for the high false-positive rates due to multiple comparisons.

Table 4. Significantly altered genes following treatment with 0.3 and 3 μ M PEITC and 0.3 and 3 μ M sulforaphane (SUL) in HMEC Cells

Coagulation factor III (thromboplastin, tissue factor)	TF
Fibronectin leucine rich transmembrane protein 1	FLRT-1
Interleukin 6 (interferon beta 2)	IL-6
Mucin 1, transmembrane	Episialin
Transforming growth factor, alpha	TGF- α
Metallothionein-III	MT-3

Interestingly, a number of other genes were altered by the low concentrations of PEITC and/or sulforaphane.

Table 5. Significantly altered genes following treatment with 0.3 and 3 μ M PEITC and sulforaphane (SUL) in MCF-7 Cells

Gene		PEITC	SUL
Androgen receptor	AR		0.3
Alpha-2-glycoprotein,zinc	AZGP1		0.3
Collagen, type VI, alpha 1	COL6A1		0.3
GNAS complex locus	GNAS1		0.3
Gelsolin	gelsolin		0.3
Heat shock 27kDa protein 1	HSP28/HSP27		0.3
Inhibitor of DNA binding 2	ID2	0.3	
V-jun sarcoma virus 17 onocogene homolog	V-jun	0.3	
Keratin,hair,basic	KRTHB6		0.3
Nerve growth factor receptor	NGFR		0.3
Phosphatase and tensin homolog	PTEN		0.3

Ribosomal protein L27	RPL27		3
S100 calcium binding protein A2	CaN19		3
Secretoglobin, family A2, member 2	SCGB2A2	0.3	
Solute carrier family 7, member 5	CD98		3
Small proline-rich protein 1B	SPRR1B		3
Stannocalcin 2	STC2		3
Thrombospondin 2	THBS2		0.3
Thrombospondin 4	THBS4		0.3

Conclusions: The studies are complete and are being currently evaluated to determine targets important in the mechanism of action of PEITC and sulforaphane in breast cancer prevention and/or treatment. A number of the changes appear to be important in cell cycle regulation and apoptosis, and these changes are concentration dependent. Of particular importance changes were predominately observed at the low concentration (0.3 μ M) investigated, and often not at the higher concentration (3 μ M).

Other

Review Paper.

We have written a review on **Isothiocyanates and Cancer Prevention** for a book entitled Nutrition and Cancer Prevention. This is currently in press.

KEY RESEARCH ACCOMPLISHMENTS-2003-05

- The pharmacokinetics of PEITC have been determined in rats. We have shown for the first time that the clearance of PEITC is dose dependent, and that following oral administration of PEITC, the bioavailability is high. Due to the high systemic availability of PEITC, it can be administered in the diet.
- Our data support our hypothesis that PEITC alters estradiol concentrations both in human breast cancer cells and in rat liver.
- Low concentrations of PEITC (0.3 μ M) alter estrogen-related gene expression in human breast cancer MCF-7 and normal human mammary cells. Interestingly, changes were predominantly observed only after exposure to low concentrations (0.3 μ M) and fewer changes were observed after exposure to a 3 μ M concentration. These studies provide new information on the estrogen-related effects of PEITC on gene expression in normal mammary epithelial and breast cancer cells.

REPORTABLE OUTCOMES

Manuscripts:

Morris ME and Telang U. **Isothiocyanates and Cancer Prevention** in Nutrition and Cancer Prevention, Awad AB and Bradford PG (editors), Taylor and Francis, to be published 2005

Ji Y and Morris ME. Pharmacokinetics of dietary phenethyl isothiocyanate in rats, Pharm Res, accepted.

Abstracts (in Appendix):

The Pharmacokinetics of Phenethyl Isothiocyanate (PEITC) in Rats. Ji Y and Morris ME, to be presented at the American Association of Pharmaceutical Scientists Meeting, Baltimore MD, November 2004.

Graduate Students Participating in this Research as a part of their educational program during 2003-05:

Yan Ji, Ph.D. candidate (graduated, 2005)

Urvi Telang, Ph.D. candidate (degree expected, 2008)

Professional student Participating in the Research as a part of her educational program during 2003-04:

Elizabeth Scott-Ramsay (PharmD awarded, May 2004)

Research technician: Yang Qu

CONCLUSIONS

1. The pharmacokinetics and bioavailability of PEITC were determined in rats. The clearance of PEITC was dose dependent, but PEITC exhibited excellent bioavailability following oral administration.
2. PEITC exposure decreases the concentrations of 17β -estradiol in human breast cancer cells.
3. PEITC administration decreases the concentrations of 17β -estradiol in rat liver after its oral administration.
4. PEITC treatment alters estrogen-related gene expression in both human mammary epithelial cells and human breast cancer MCF-7 cells at low ($0.3 \mu\text{M}$) concentrations.

Appendix

The Pharmacokinetics of Phenethyl Isothiocyanate (PEITC) in Rats

Ji Y and Morris ME. Department of Pharmaceutical Sciences, University at Buffalo, Amherst NY 14260.

Purpose: Phenethyl isothiocyanate (PEITC), a dietary compound in cruciferous vegetables, has chemopreventive properties and is being investigated in Phase I clinical studies. The pharmacokinetics of PEITC are largely unknown. The objective of this study was to examine the pharmacokinetics of PEITC in rats following oral and intravenous administration.

Methods: Male Sprague-Dawley rats were administered PEITC at doses of 2, 10, 100 or 400 $\mu\text{mol/kg}$ i.v. or 10, 100 or 400 $\mu\text{mol/kg}$ orally. PEITC was prepared in 15% hydroxy-propyl- β -cyclodextrin. Plasma samples were collected at 5, 15, 30 min and 1, 2, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h and analyzed by a LC/MS/MS assay. Pharmacokinetic data were analyzed by WinNonlin for non-compartmental analysis and ADAPT II for compartmental analysis.

Results: With an increase in the PEITC dose, elimination half-life ($t_{1/2}$) and time to C_{max} (t_{max}) increased, maximal plasma concentrations (C_{max}) increased but not proportionally, and oral bioavailability (F) decreased. At the highest dose, Cl was decreased while V was increased. The plasma concentration profile of PEITC after i.v. administration can be well characterized by a three-compartment model with Michaelis-Menten elimination and distribution.

Dose ($\mu\text{mol/kg}$)	2	10	100	400
$t_{1/2}$ (h)	3.52 ± 0.35	6.92 ± 3.73	9.19 ± 0.83	13.1 ± 2.0
Cl (L/h/kg)	0.70 ± 0.17	0.68 ± 0.29	0.36 ± 0.18	0.50 ± 0.04
V (L/kg)	3.52 ± 0.63	7.82 ± 5.66	4.94 ± 2.84	9.46 ± 2.06
C_{max} (μM)		9.2 ± 0.6	42.1 ± 11.4	48.0 ± 5.9
t_{max} (h)		0.4 ± 0.1	2.0 ± 1.0	2.3 ± 1.2
F (%)		115	92.4	63.8

Conclusions: PEITC is a dietary component with high oral bioavailability and low clearance in rats. Nonlinear elimination and distribution is evident at high doses.

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Isothiocyanates and Cancer Prevention

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Abbreviations:

Isothiocyante (ITC)
Sulforaphane (SF)
Phenethyl isothiocyante (PEITC)
Naphthyl ITC (NITC).
Allyl isothiocyante AITC
Benzyl isothiocyante BITC
Glutathione S-transferase GST
Daunomycin (DNM)
Vinblastine (VBL),
Odds ratio (OR)
Risk Ratio (RR)
4-(methylnitrosamino)-1-(3-pyridyl)1-butanone (NNK)
Benzo (a) pyrene (BaP),
Mitogen-Activated-Protein-Kinase (MAPK)

I. Introduction

Extensive epidemiological evidence supports the contention that high fruit and vegetable consumption is associated with a reduction in the incidence of cancer. Moreover, it is well established that consumption of cruciferous vegetables is inversely associated with the risk of cancer of the lung, colon, stomach and prostate (1-3). In the mid 1980s, studies in rats demonstrated that isothiocyanates (ITCs) (Figure 1), which occur widely as conjugates in *Brassica* and other vegetables of the family Cruciferae (e.g. cabbage, cauliflower, brussels sprouts, watercress, broccoli, kale) and the genus *Raphanus* (radishes and daikons), inhibit the metabolic activation of a variety of carcinogens that occur in tobacco products and the diet, suggesting chemopreventive effects of ITCs (4, 5). Organic ITCs ($R-N=C=S$) occur in plants as thioglycoside conjugates known as glucosinolates. Damage to plant cells, such as from cutting and chewing, releases myrosinase (β -thioglucoside glucohydrolase) that catalyzes the hydrolysis of glucosinolates and the formation of ITCs by a Lossen rearrangement (Figure 2). The microflora in the intestinal tract also acts as a source for the hydrolysis of glucosinolates to ITCs in humans (6).

Consuming normal amounts of vegetables such as watercress or broccoli releases tens of milligram amounts of ITCs (7). Glucosinolate levels have been estimated to be as high as 180mg/g in some vegetables (7). High levels of sulforaphane (SF) and phenethyl isothiocyanate (PEITC), the two most common ITCs in the diet are detected in plasma following the consumption of broccoli and watercress respectively (8, 9). The estimated isothiocyanate/glucosinolate levels in common cruciferous vegetables are given in Table 1. It has been estimated that the consumption of 100 g of broccoli could release 40 μ moles of the ITC sulforaphane (10) while consumption of about 30 g of watercress releases about 46.5 μ moles of PEITC (11). Additionally, ITCs are constituents of numerous herbal supplements including

Daily Cruciferous Plus®, Broccoli Sprouts®, BioBasics®, Fruit and Veggie Tabs®, and NutriHealth MultiPlus®.

More than 20 natural and synthetic ITCs have been shown to block carcinogenesis. The capacity for organic isothiocyanates to block chemical carcinogenesis was first recognized over 30 years ago with naphthyl ITC (NITC). Recent studies describing the effect of ITCs in vitro, in vivo and in clinical studies are described later in this chapter. Current mechanisms proposed for the anticarcinogenic effects of ITCs include: (1) inhibition of Phase I enzymes converting procarcinogens to highly reactive electrophilic carcinogens; (2) induction of Phase II enzymes inactivating carcinogens and promoting their excretion; and (3) induction of apoptosis of cancer cells (12, 13).

II. Bioavailability and Pharmacokinetics of ITCs

Absorption of isothiocyanates is rapid, and the parent compound can be detected in the blood minutes after administration. The pharmacokinetics of PEITC both in rats and humans have been evaluated in our laboratory: PEITC has high oral bioavailability and low clearance (0.70 ± 0.17 L/h/kg at the lowest dose of 2 $\mu\text{mol/kg}$) in rats (Ji, Y and Morris, ME, unpublished). Nonlinear elimination and distribution were evident at high doses. In humans, following ingestion of the vegetable watercress at a 100 g dose, the mean C_{max} value was 928 nM and the half-life was 4.9 hours (Fig. 3). Average oral clearance (clearance/availability) was 490 ml/min, suggesting a low clearance (14). A recent evaluation of a high dose of sulforaphane (50 μmol) in rats reported high concentrations in plasma (C_{max} of 20 μM) and a half life of about 2.2 hours (15). Therefore, in contrast to dietary components such as the flavonoids, oral clearance of these ITCs is low and bioavailability is excellent, indicating greater exposure to the parent compound in the intestine, liver, and systemically, than observed with other dietary chemicals.

In humans, the conjugation and excretion of ITCs is mainly catalyzed by glutathione S-transferase M1 (GSTM1) and GSTT1 (16), although a variety of other GSTs, including GST A1, P1, M2 and M4 are also involved to a minor extent. GSTM1 and GSTT1 exhibit significant polymorphisms in humans: the incidence of homozygous null deletion is approximately 50% for GSTM1 in white subjects in the USA, as well as in Japanese and Chinese subjects; for GSTT1 the incidence is 12-16% in German and English subjects and 60-64% in Chinese and Koreans (17). Studies examining the correlation of ITC intake obtained through vegetable consumption and GSTM1 and GSTT1 genotypes among various populations have also suggested that lung cancer risk and colorectal adenomas were decreased among persons genetically deficient in GSTM1 and/or GSTT1, although GSTM1 deficiency appears to be more important (1, 18-20). In those subjects with detectable levels of ITCs in their urine and a GSTM1 deficiency, there was a 64% decrease in the risk of developing lung cancer (20). These studies suggest the importance of the polymorphic expression of GSTs in determining the systemic concentrations and efficacy of ITCs.

III. In Vitro Studies

Inhibition of cell growth and modulation of the cell cycle have been reported for allyl isothiocyanate (AITC) (10 μ M), benzyl isothiocyanate (BITC) (5 μ M) and PEITC (2.5 μ M) in HeLa cells (21), and similar effects have been reported for sulforaphane (15 μ M) in HT-29 cells. Similar results have been reported in other cell lines including human leukemia HL60 and prostate cancer cells lines LNCaP and DU-145 (reviewed by (22)). A comprehensive study of the effect of isothiocyanates on the growth of different cancer cells reported that AITC, BITC and PEITC were able to inhibit cell growth (23).

The cytotoxicity of PEITC, BITC, NITC and sulforaphane, as well as the cytotoxicity of the chemotherapeutic agents daunomycin (DNM) and vinblastine (VBL), have been evaluated in

human breast cancer MCF-7 and human mammary epithelial MCF-12A cells (24). IC_{50} values for BITC, PEITC, NITC and sulforaphane were 5.95 ± 0.10 , 7.32 ± 0.25 , 77.9 ± 8.03 and 13.71 ± 0.82 μM in MCF-7 cells. The corresponding IC_{50} values for DNM and VBL in MCF-7 cells were 7.12 ± 0.42 μM and 0.106 ± 0.004 μM (mean \pm SE). Values for BITC, PEITC, NITC and sulforaphane in MCF-12A cells were 8.07 ± 0.29 , 7.71 ± 0.07 , 33.63 ± 1.69 , and 40.45 ± 1.25 μM , respectively. BITC and PEITC can inhibit the growth of human breast cancer cells as well as human mammary epithelium cells, at concentrations similar to the chemotherapeutic drug DNM. Sulforaphane and NITC exhibited higher IC_{50} values. These concentrations are 4-6 fold lower than the IC_{50} for the isoflavonoid, genistein, a compound that has also been studied in MCF-7 cells. Genistein has been reported to have the lowest IC_{50} among the dietary flavonoids tested in MCF-7 cells (25).

While some studies have demonstrated similar cytotoxicity in cancer and normal cells (24, 26), others have reported differences. A synthetic isothiocyanate MTBITC has shown selective action against human leukemia cells, and almost no effect on lymphocytes (27). AITC also has an inhibitory in the human prostate cancer cell lines, while normal cell line PrEC remained unaffected by the same exposure (28).

The cytotoxic effects of isothiocyanates are apparent even after shorter exposures. Comprehensive time-dependent studies of the effect of isothiocyanates on the growth of different cell lines reported that a number of ITCs, including AITC, BITC, PEITC and NITC, were able to inhibit cell growth after 2 or 3 hour exposures, producing IC_{50} s similar to those for exposure for 48-72 hours, indicating that short-term exposure is sufficient to produce observable effect in vitro (23, 24).

IV. In Vivo Studies with Animal Cancer Models

The anticarcinogenic activities of ITCs have been demonstrated in a number of carcinogen-induced cancer models in vivo. Over 20 natural and synthetic ITCs have demonstrated cancer preventive properties in animals treated with chemical carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines. For example, ITCs can inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK)-induced carcinogenesis by inhibiting the microsomal metabolism of NNK to reactive species that form methyl and pyridyloxobutyl adducts in DNA (29-31). Carcinogen inhibition was seen in a number of different organs such as lung, liver, fore-stomach, mammary gland and colon. BITC has shown effectiveness in mammary gland cancer, while PEITC has been effective in carcinogen induced models of mammary, lung, oral as well as esophageal cancer in rats and mice (32, 33). Studies have shown that sulforaphane is effective in prevention of azoxymethane-induced colonic cancer (5). Table 4 summarizes the results of recent studies on carcinogen-induced cancer models in rodents.

V. Clinical Studies

Several epidemiological studies have reported a correlation between the intake of Brassica vegetables and the risk of cancers in humans. Although this inverse correlation cannot be attributed solely to isothiocyanates, as other constituents such as vitamins, folic acid and fiber may also play a role in the reduction of cancer risk, newer studies quantifying the amount of ITCs in the biological samples have provided evidence that ITCs are important in cancer prevention.

Epidemiological studies in lung cancer risk have shown associations between consumption of ITCs and reduced risk after the smoking status of the subject is taken into account. A case control study in Chinese women reported an odds ratio (OR) of 0.31 for

smokers with high ITC intake, and an OR of 0.70 for non smokers with comparable ITC intake. (19).

A breast cancer study with 720 cases and 810 controls reported that the consumption of broccoli, a vegetable rich in sulforaphane, is inversely associated with breast cancer risk in premenopausal women and that the association was not significant in post-menopausal women. When they measured cancer risk and total cruciferous vegetable intake, the correlation was not significant (34). A study in Chinese women evaluated the correlation between urinary isothiocyanates levels (determined by a cyclocondensation derivatization of the total isothiocyanates and dithiocarbamates in the urine (35)) to breast cancer risk. The study, performed on 337 cases and matched controls, reported a 50% reduction in the cancer risk for the highest quartile of ITC consumption (36).

A prospective case control study in male subjects reported that the section of the population with the highest intake of cruciferous vegetables had a lower incidence of bladder cancer. Detailed studies into the risks associated with individual cruciferous vegetables showed that cabbage and broccoli were able to reduce the risk, independent of the intake of other crucifers (37).

A case control study on Chinese subjects compared the dietary intake of cruciferous vegetables in 213 cases of colorectal cancer with 1194 controls. When their data was categorized by ITC consumption, a non significant association was seen between high intake and colorectal cancer risk. However, there was a significant inverse correlation seen between colorectal cancer risk and high intake for individuals with null genotypes for GSTM1 and T1 (38), suggesting that the higher plasma concentrations of ITCs that would be expected in individuals with null genotypes may be responsible for this significant effect.

Thus, while some studies have reported that the association between crucifer consumption and cancer risk was not significant, other studies have shown strong associations

after adjustments for lifestyle practices such as smoking and for genotypes of glutathione S transferase. Methodological differences can produce differences in the results of epidemiological studies. Most studies categorize the subjects by crucifer intake levels and Table 5 includes recent studies containing data for the highest crucifer intake groups. The incorporation of indicators of ITC consumption (such as the quantitation of total dithiocarbamates in urine) has resulted in the demonstration of significant inverse correlations between diet and cancer risk even with smaller studies, indicating the importance of verifying ITC exposure (36) Reference for urinary marker

VI. Mechanisms of action of isothiocyanates

Inhibition of Phase I enzymes:

Isothiocyanates have been shown to inhibit rat and human cytochrome P-450 (CYP) isoforms, important for the activation of procarcinogens. CYPs 1A1, 1A2, 2B1, 2E1 and 3A4 are inhibited by ITCs, through competitive, noncompetitive or mixed inhibition (39). Of these isoforms, 1A1 and 2E1 play the most important role in the activation of carcinogens. Table 6 presents studies in which ITCs have shown to exert inhibitory actions on CYPs. Structural activity relationship studies have shown that arylalkyl isothiocyanates with 6 carbon chains can cause maximum inhibition of CYP enzymes in rat liver microsomes (40).

Human and rodent studies have demonstrated that PEITC blocks metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo(a)pyrene (BaP), major lung carcinogens in tobacco smoke, via CYPs, resulting in increased urinary excretion of detoxified metabolites, suggesting inhibitory effects on CYP1A1, 1A2 and 2B1 (29, 31, 40). In humans, watercress ingestion resulted in a reduction in the levels of oxidative metabolites of acetaminophen, which was attributed to inhibition of oxidative metabolism by CYP 2E1 (41), and enhancement in the area under the plasma concentration-time curve (AUC) of

chlorzoxazone, a clinical probe for CYP2E1 (42). Watercress consumption in humans was shown to inhibit the metabolism of the tobacco specific carcinogen NNK (4-methylnitrosamino)-1-(3-pyridyl)-1-butanone), possibly by the inhibition of phase I enzymes like CYP1A2 (43).

Recently, using microsomes from baculovirus-infected insect cells expressing human CYP isoforms, PEITC was found to competitively inhibit CYP1A2 and 2A6, noncompetitively inhibit CYP2B6, 2C9, 2C19, 2D6, and 2E1, and inhibit CYP3A4 following a mixed-type of competitive and noncompetitive inhibition (44, 45) However, controversial data exist. After PEITC administration to mice, CYP2E1 level increased in liver and lung microsomes (31). Administration of PEITC to rats has been found to cause modest induction of CYP1A1 and 1A2 both in protein expression and metabolic activity (46), different from the observation in baculovirus systems. A human study failed to show alteration of coumarin metabolism, a substrate for CYP2D6, after watercress consumption (47).

The Phase II enzyme system:

Phase II enzymes are generally referred to as detoxifying enzymes, transferring hydrophilic endogenous substances such as glucuronic acid, sulfate or glutathione to phase I metabolites, or parent molecules. The hydrophilic molecules thus formed are more easily cleared from the body than their lipophilic parent compounds. ITCs have been classified as monofunctional inducers of Phase II enzymes, i.e. they induce phase II enzymes without inducing Phase I enzymes.

A number of studies have demonstrated the Phase II inducing properties of ITCs both in vivo and in vitro. An in vivo study of a mixture indole-3-carbinol, PEITC and 1-isothiocyanato-3-(methylsulfinyl)-propane (a glucosinolate breakdown product) in F344 mice at dietary doses showed increase in the pancreatic mRNA levels for the enzymes quinone reductase (QR) and glutathione S transferase (GST) by 3.1 and 7.1 fold respectively. Another comprehensive in vivo

study of seven isothiocyanates was conducted by Munday and compared the levels of induction of QR and GST of the ITCs.

Sulforaphane has been reported to be the most potent phase II enzyme inducer, while others like PEITC and AITC also showed significant induction of the enzymes. Sulforaphane induced the transcription of UGT1A1 and GSTA1 in Caco2 cells and produced a synergistic induction effect on UGT1A1 with apigenin. Sulphoraphane induced the phase II enzyme GSTA1 at the transcriptional levels in human prostate cancer cell lines, HepG2, HT29 and enterocytes. In HepG2 and HT29 cells, doses of 0.3-30 μ M of sulphoraphane significantly induce the transcription of GSTA1 and UDP glucuronosyl transferase, and exhibit a 2.8 fold increase in the formation of bilirubin glucuronide, indicating increased activity of the UDP enzyme. A human study, on jejunum enterocytes has also reported that there was an increase in the induction of these enzymes 2.0 and 2.4 fold respectively.

Other isothiocyanates, 7-methylsulfinylheptyl ITC and 8-methylsulfinyloctyl ITC present in watercress have also been reported to have potent inducing activity towards quinone reductase (48). BITC induced GSTP1 isoform in rat liver epithelial cells RL34 cells and increased in NQO activity in LS-174 human colon cell line (44). PEITC increases the activities of UDP-glucuronosyltransferase (UGT), GST and NAD (P) H: quinone oxidoreductase 1 (NQO1) (48). PEITC treatment of LS-174 human colon cells produced an increased protein expression of NQO1 and γ -glutamylcysteine synthetase (49). Following watercress ingestion in smokers, there was an increased urinary elimination of the glucuronides of cotinine, suggesting an increased glucuronidation (29).

Recent studies have suggested that ITCs induce GST, NAD (P) H: quinone oxidoreductase and γ -glutamylcysteine through activating genes via the antioxidant/electrophile response element (ARE/EpRE), located upstream region of genes that code for these enzymes.

ITCs dissociate the cytoplasmic-anchoring protein Kelch-like ECH-associated protein 1 (Keap I) from the transcription factor Nrf2, allowing the latter to translocate to the nucleus and to form Nrf2/Maf heterodimers, which bind ARE/EpRE and activate transcription of genes coding the enzyme (12, 50). One proposed mechanism for the ITC-mediated effect on Keap I is ITC binding to the ATP binding domain present in Keap I. Keap I is a 624 amino acid protein that contains 25 cysteine residues, 9 of which are expected to have highly reactive sulfhydryl groups because they are close to 1 or more basic amino groups (51).

Pathways of apoptosis:

Most ITCs inhibit cell growth by arresting the G2/M or the G1 cycle. The induction of apoptosis occurs via the activation of intracellular signaling pathways such as the MAPK and the caspase pathway. The MAPKs (ERK, JNK and p38) convert various extracellular signals to intracellular responses through a series of phosphorylation pathways. Caspases are cysteine proteases, which are activated as a result of binding of death ligands to death receptors. The balance of anti-apoptotic Bcl-2 and Bcl-XL versus pro-apoptotic (Bad and Bax) proteins on the cell is also an important determinant of stress required to initiate apoptosis.

Studies into the mechanism of in vitro apoptosis induction have shown that while the action of sulforaphane and AITC is linked to an increase in caspase activity and the expression of pro-apoptotic proteins in the Bcl-2 family (28, 52), PEITC may cause the activation of the MAPK pathways, with JNK being strongly activated. Components such as p53 dependent pathways have also been reported to be involved in the process (53, 54). In prostate cancer cell lines, PEITC has been shown to increase apoptosis by the reduced expression of Bcl-2 and Bcl-XL, and an increase in caspase activity.(28). A proposed sequence of events in apoptosis induced by ITCs is illustrated in a review by Thornalley (13).

Conclusions

Isothiocyanates are widely present in the human diet. Epidemiological as well as experimental data has shown that isothiocyanates have the potential to reduce the risk of cancer. Isothiocyanates modulate enzyme activity *in vivo* as well as *in vitro* and have also been shown to induce apoptosis and inhibit cell growth *in vitro* in a number of human cancer cell lines. Their properties of chemoprevention have made the compounds attractive candidates for development as drugs. PEITC has entered a Phase I clinical trial for the compound in its ability to prevent lung cancer in smokers (55). Development of synthetic more potent analogues of ITCs that can be developed as drugs has also been undertaken (56). As ITCs enter clinical use, the influence of the genetic polymorphism of GST on the chemopreventive properties of ITCs needs to be further studied.

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Table 1. Isothiocyanate content in the diet.

Vegetable	Glucosinolate (GL) or ITC content	Reference
Raw Cabbage	0.108 $\mu\text{mol/g}$ (PEITC)	(57)
Mustard	10.8 $\mu\text{mol/g}$ (AITC) 3.2 $\mu\text{mol/g}$ (PEITC)	(57)
Broccoli Sprouts	8 $\mu\text{mol/g}$ ITCs	(58)
Watercress	6.5 $\mu\text{mol/g}$ ITC	(59)
Brussels Sprouts	15.84 mol/g (total Glucosinolate) 8.7 mol/g (sinigrin)	(60)

Table 2. In vitro cytotoxic effects of isothiocyanates

Table 2. In vitro cytotoxic effects of isothiocyanates				
Isothiocyanate (Treatment)	Cell type Cell line	Cell Density/Survival	Apoptosis Induction	Reference:
Inhibition				
Sulforaphane				
(24 hours 50 μM)	human prostate cancer LNCaP	50%	50%	(61)
(24 hours 15 μM)	human colon carcinoma HT29	25%	75%	(62)
(48 hours)	mouse breast cancer F3II	IC ₅₀ : 8 μM		(63)
(48 hours)	human breast cancer MCF- 7	IC ₅₀ : 13.7 μM		(64)
BITC				
(48 hours)	human breast cancer MCF- 7	5.95 μM		(64)
(24 hours)	human pancreatic cancer BxPC-3	IC ₅₀ : 8 μM	3 fold (5 μM)	(65) (66)
(24 hours)	human leukemia Jurkat T	IC ₅₀ : 6 μM	6 fold (5 μM)	
PEITC (24 hours 5 μM)	Human prostate cancer PC-3	75%	3 fold	(67)
(3 hours)	human bladder carcinoma UM-UC-3	IC ₅₀ : 22.0 μM		(68)
(24 hours)			5.9 fold (15 μM)	(68)
MBITC				
(24 hours 10 μM)	human leukemia Jurkat T	+2.8 fold G2 phase cell arrest	2.4 fold	(27)
AITC				
(24 hours 40 μM)	Human prostate cancer LNCaP	40%	3 fold	(28)
(3 hours)	Human colon cancer HT-29	IC ₅₀ : 5.9 μM		(23)

Table 3. Effects of ITCs on carcinogen-induced cancer in animal studies.

ITC (Treatment)	Animal	Model	Carcinogen (Dose)	Dosing	% rats with tumors (test vs. control)	Reference
BITC (50mg)	Sprague Dawley rats f	Mammary	DMBA (12 mg)	Oral: Single Dose 4 hours before DMBA	8/77	(69)
(0.017 mmol/g diet)				Diet: DMBA+BITC 10 weeks	63/100	(69)
(5.0 mg/g diet)	ICR/Ha mice f	Forestomach	DMBA (0.05mg/g diet)	Diet: DMBA+BITC 4 weeks	5/87	(69)
PEITC (55mg)	Sprague Dawley rats f	Mammary	DMBA (12 mg)	Oral: Single Dose 4 hours before DMBA	43/100	(69)
(5.5 mg/g diet)	ICR/Ha mice f	Forestomach	DMBA (12 mg)	Diet: DMBA+PEITC 4 weeks	12/93	(69)
(3 μ mol/g diet)	F344 rats m	Lung	NNK (1.76 mg/kg body wt)	Diet: PEITC 21 weeks + s.c NNK at end of 1 st week,	43/80	(32)
(5 μ mol)	A/J mice f		NNK (10 μ mol)	Oral: PEITC 2 hours before NNK i.p	62/100 (tumor multiplicity in each rat)	(70)
(50 mM)	Syrian hamster m	Buccal pouch	NMBA (50mM)	Topical 24 weeks	6%	(71)
Sulforaphane (20 μ mol)	F344 rats m	Colon	AM (15 mg/kg body wt)	Oral: 3 times a week for 8 weeks, weekly s.c axozymethane weekly for 2 weeks,	40/100	(5)
(5 μ mol)					42/100	(5)

M: male; F: Female; DMBA: 7,12-dimethyl-benz[a]anthracene; NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-butanone

AM: axozymethane; NMBA: N-nitrosomethylbenzylamine

Table 4. Clinical studies

Cancer Investigated	Population	Study Design	OR or RR for high intake groups.	Comment	Reference
Lung cancer	Chinese	223 Cases	0.31 (smokers)		(72)
	Women	187 Controls	0.70 (non smokers)		
	American	503 cases	1.09 (smokers)	Data in column are for subjects null for GST T1 and M1. These showed least OR.	(73)
		465 controls	1.08 (former smokers)		
Breast cancer	American	740 Cases 810 control	0.6 (premenopausal)	Broccoli intake was measured. No significant associations for GST polymorphs or post menopausal women	(34)
	Chinese	1459 Cases 1556 controls	0.5	Reports that association was seen in pre and post menopausal women	(36)
Bladder Cancer	Males	47909 Subjects	RR 0.49	Lowest incidence of cancer observed in the group with highest intake of cruciferous vegetables.	(37)

OR: Odds Ratio; RR: risk Ratio

Table 5. Phase I enzyme modulation by isothiocyanates

ITC	System	Enzyme	Reaction monitored	Result	Reference
PEITC	RLM	CYP2E1	NDMA demethylation	IC ₅₀ : 8.3 µM	(74)
		CYP1A2	MROD	IC ₅₀ : 54 µM IC ₅₀ : 1.2 µM	(75)
	HLM	CYP2B1/2	PROD	IC ₅₀ : 4.6 µM	(76)
		CYP1A2	NNK keto- alcohol formation		
	R-CYP (human)	CYP1A2	Phenacetin-o-deethylase	Ki: 4.5 µM	(39)
Sulforaphane 10 µM	HH	CYP3A4	Changes in mRNA expression	-2.5 fold	(77)
5 µM for 24 hours	RH	CYP1A1	EROD	-80%	(77)
Red cabbage juice	Rat (oral; RLM activity measured)	CYP1A2	MROD	+2.61 fold	(78)

NDMA: N-dimethylnitrosamine, NNK: 4-(-methylnitrosamino)-1-(3-pyridyl)-1-butanone

MROD: methoxyresorufin dealkylation, EROD: ethoxyresorufin dealkylation, PROD: pentoxyresorufin dealkylation

RLM: Rat Liver Microsomes; HLM: Human Liver Microsomes; RH: Rat Hepatocytes; HH: Human Hepatocytes; R-CYP: Recombinant cytochrome P450. Positive sign indicates induction and negative sign indicates inhibition

Table 6: Phase II enzyme induction by isothiocyanates

Isothiocyanate treatment	Cell line/Tissue	Enzyme in System	Induction effect	Reference
PEITC 5µM	Human colon cell line	QR	+140%	(79)
Sulforaphane 10µM	CaCO2 cells	UGT1A1, GST1A	+2 fold	(80)
10µM	Human prostate cancer cell	GST1A	+1.7 fold	(81)
1 µM	Human lymphoblastoid cells	QR	+2 fold	(82)
40 µmol/kg/day	Rat duodenum	GST	+2.48 fold	
7-methylsulfinylheptyl ITC 0.2µM	Hepa 1c1c7 cells	QR	+2 fold	448)
6- methylsulfinylhexyl ITC (15 µmol/day)	ICR mice (liver)	GST	+2 fold	(84)
AITC 40 µmol/kg/day	Rats (forestomach)	QR	+1.5 fold	(83)
BITC 10µM	RL34 rat liver cells	GSTP1	+2.3 fold	(44)

QR: Quinone Reductase GST: Glutathione S transferase

Figure legends.

Figure 1. Chemical structures of common isothiocyanates.

Figure 2. Hydrolysis of glucosinolates and the formation of ITCs by a Lossen rearrangement.

Figure 3. Plasma concentration versus time profile of PEITC in humans following the consumption of 100 g watercress. Data are expressed as mean \pm SD, n = 4; closed circles represent the measured concentration and the line represents the predicted concentration fitted by compartmental model analysis using WinNonlin Version 2.1 (Pharsight, Mountainview, CA).

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Figure 4. Cytotoxicity in human breast cancer MCF-7 cells.

The effect of varying concentrations of (A) BITC (B) sulforaphane and (C) PEITC on cell growth of MCF-7/Adr cells following exposure times of (▼) 1 hour, (■) 2 hours, (▲) 3 hours, (◆) 6 hours and (●) 48 hours. Each data point represents mean \pm SE from 4 wells in one representative study. The study was repeated two to four times. Reproduced from Exp Biol Med (24) with permission from the Society for Experimental Biology and Medicine.

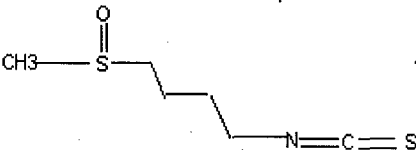
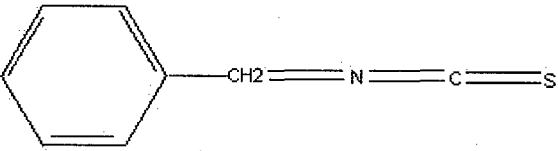
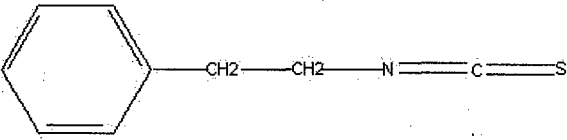
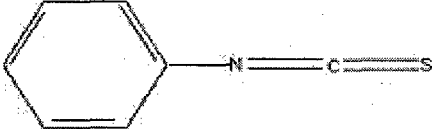
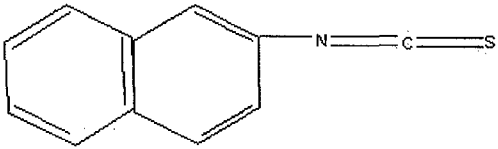
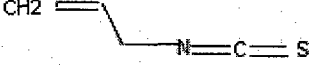
Name	Structure
Sulforaphane	
Benzyl isothiocyanate	
Phenethyl isothiocyanate	
Phenyl isothiocyanate	
Naphthyl isothiocyanate	
Allyl isothiocyanate	

Figure 2.

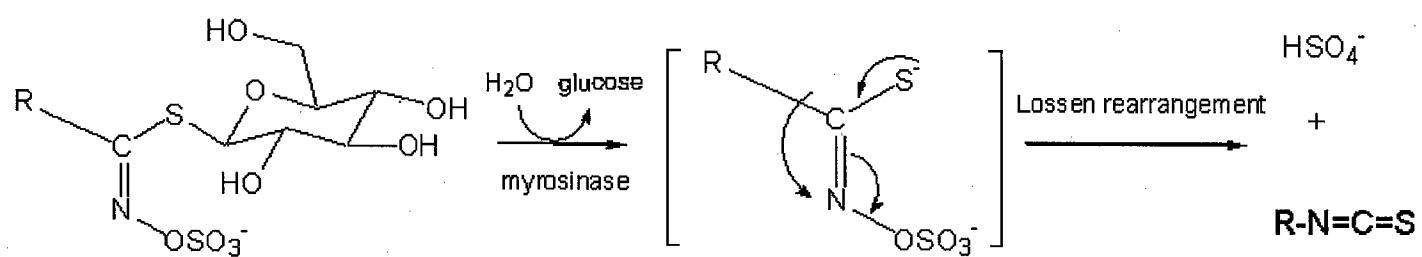


Fig. 3

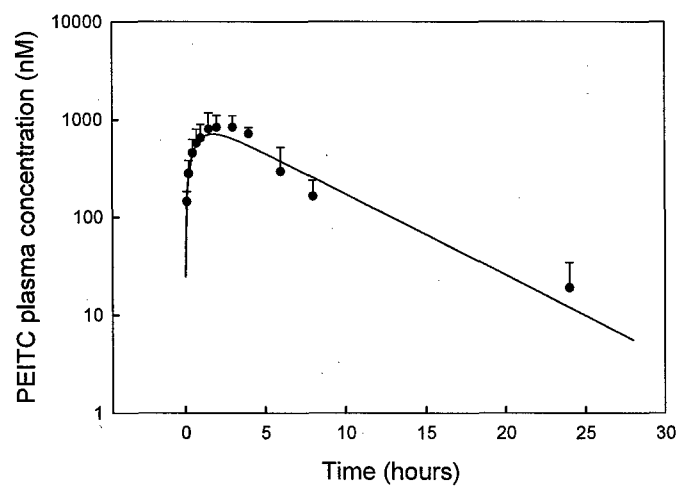
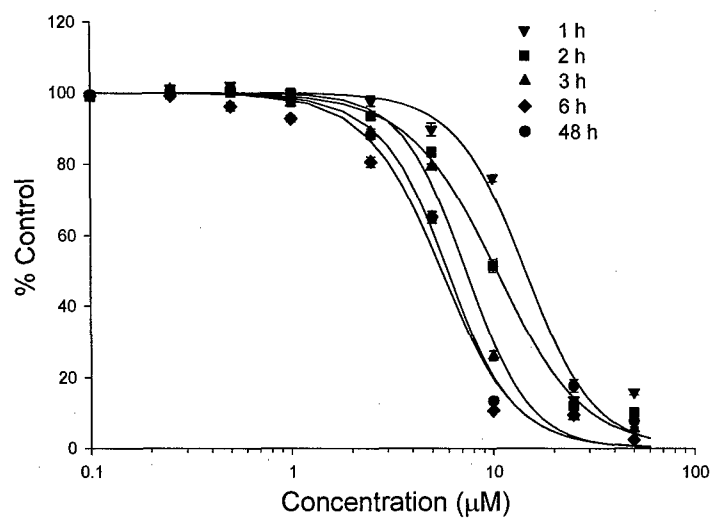
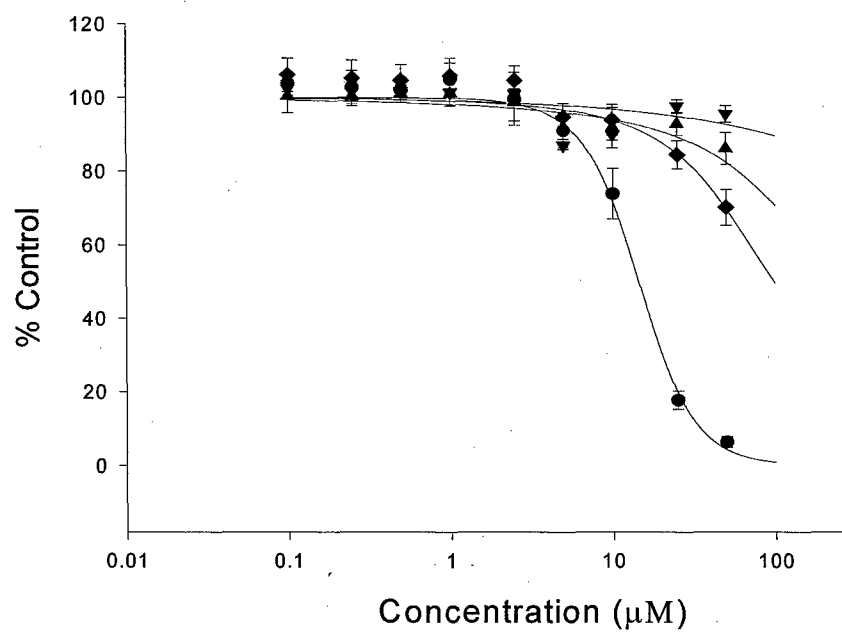


Fig. 4.

A



B



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Pharmacokinetics of Dietary Phenethyl Isothiocyanate in Rats

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ABBREVIATIONS

AUC, area under the plasma concentration vs. time curve; BCRP, breast cancer resistance protein; C_{\max} , maximal plasma concentration; Cl, clearance; F, bioavailability; f_u , the unbound fraction; ITC, isothiocyanate; i.v., intravenous; k_a , absorption rate constant; k_d , degradation rate constant; MRP, multidrug resistance-associated protein; PEITC, phenethyl isothiocyanate; PEITC-SG, glutathione conjugate of PEITC; RT, room temperature; t_{\max} , time to reach C_{\max} ; $t_{1/2}$, elimination half-life; $t_{1/2,d}$, degradation half-life; V_{ss} , volume of distribution.

ABSTRACT

Purpose. Phenethyl isothiocyanate (PEITC) is a dietary component present in cruciferous vegetables and reported to have chemopreventive properties. Previous reports of PEITC pharmacokinetics have measured total ITC (PEITC and its metabolites) in plasma. Our objective was to examine the dose-dependent pharmacokinetics and oral bioavailability of unchanged PEITC, as well as its pH- and temperature-dependent stability and its serum protein binding.

Methods. Stability was studied at different pH values at room temperature and 4 °C. Protein binding was determined by equilibrium dialysis. For the pharmacokinetics study, male Sprague-Dawley rats were administered PEITC at doses of 2, 10, 100 or 400 µmol/kg intravenously, or 10 or 100 µmol/kg orally. Plasma samples were analyzed by LC/MS/MS. Pharmacokinetic analysis was conducted by WinNonlin and ADAPT II.

Results. PEITC was stable in aqueous buffers at pH 7.4 with half-lives of 56.1 and 108 hours at room temperature and 4 °C, respectively. The free fraction of PEITC in rat serum was 0.019. The clearance (Cl) at a low dose of PEITC (2 µmol/kg) was 0.7 ± 0.17 L/h/kg with an apparent volume of distribution (V_{ss}) of 1.94 ± 0.42 L/kg. At higher doses, Cl tended to decrease while V_{ss} increased. Oral bioavailability of PEITC was 115% and 93% at doses of 10 and 100 µmol/kg. A three-compartment model with Michaelis-Menten elimination and distribution was found to best characterize the plasma concentration profiles.

Conclusions. PEITC is stable in biological samples, with increased stability under refrigerated conditions. It has high oral bioavailability, low clearance and high protein binding in rats; nonlinear elimination and distribution occurs following the

administration of high doses. This investigation represents the first report of the pharmacokinetics of dietary PEITC.

Key Words: PEITC; phenethyl isothiocyanate; stability; protein binding; pharmacokinetics; bioavailability; rats

INTRODUCTION

Isothiocyanates (ITCs) occur widely as conjugates in the genus *Brassica* of cruciferous vegetables (e.g. cabbage, cauliflower, brussels sprouts, watercress, broccoli, kale) and the genus *Raphanus* (radishes and daikons) (1). They are released by the action of myrosinase (β -thioglucoside glucohydrolase) after plant cells are damaged, such as from cutting and chewing, or by hydrolysis in the intestinal tract by microflora (1,2). More than 25 natural and synthetic ITCs have been shown to block chemical carcinogenesis effectively (3). ITCs are widely consumed by humans and it is estimated that human consumption of glucosinolates are as high as 300 mg/d (3). Phenethyl isothiocyanate (PEITC, Fig 1) is one of the extensively studied ITCs and has been launched into Phase I clinical trials for its ability to prevent cancer from occurring in high-risk individuals, such as lung cancer in smokers and ex-smokers (4). It has been demonstrated to have effective chemoprevention activity for a wide variety of tumors, and no toxicity has been observed in animal models with equivalent anticarcinogenic doses or even higher doses (5). It has been long recognized that ITCs can inhibit Phase I enzymes, including cytochrome P450 enzymes, to convert procarcinogens to highly reactive electrophilic carcinogens that can form DNA adducts; and induce Phase II enzymes, including glutathione S-transferase and quinone reductase, to inactivate carcinogens and promote their excretion (6). More recently, it has been found that ITCs could inhibit the cell cycle and induce apoptotic cell death (7-9).

The metabolism of ITCs has been studied in mice, rats, guinea-pigs and rabbits (10-14). In rats, ITCs having the $\text{RCH}_2\text{-N}=\text{C}=\text{S}$ residue, including PEITC, have been found to conjugate with glutathione first, promoted by glutathione S-transferases; the

glutathione conjugate undergoes further metabolism to form a mercapturic acid conjugate (N-acetylcysteine conjugate) (12). In guinea-pigs and rabbits, a cyclic mercaptopyruvate conjugate is the major metabolite (12), whereas mice excreted both cyclic mercaptopyruvate and the N-acetylcysteine conjugate of PEITC with the former form predominating (14). In humans, PEITC also forms a mercapturic acid conjugate, similar to the metabolite profile seen in rats (12, 15). After ingestion of watercress, a dietary source of PEITC, a dose-dependent urinary excretion of the PEITC mercapturic acid conjugate was observed (16).

Knowledge regarding the disposition and excretion of PEITC is limited, although the pharmacokinetics of ^{14}C -PEITC in rats and mice after a single oral dose has been investigated. When administered to mice by gavage at a dose of 5 μmol , ^{14}C -PEITC was readily absorbed and distributed to all major tissues, and approximately 50% of the total radioactivity was excreted within 24 hours (14). A study in rats showed similar tissue distribution, and whole blood ^{14}C radiolabel exhibited a disposition profile described by a two-compartment model with a t_{max} (time to reach maximal plasma concentration) of 2.9 h and an elimination half-life ($t_{1/2}$) of 21.7 h after an oral dose of 50 $\mu\text{mol/kg}$ (17). However, measurement of total radioactivity does not reflect the pharmacokinetic behavior of the parent compound PEITC, but that of parent and any metabolite containing the radiolabel. PEITC has been used in Phase I clinical trials to evaluate its safety and toxicity in healthy individuals, and the pharmacokinetic profiles of total ITC have been examined using a HPLC-based cyclocondensation approach; PEITC, PEITC conjugates as well as other ITCs or dithiocarbamates would be detected in this assay (4,18). No study has specifically determined the plasma concentration profiles of unchanged PEITC. However,

characterization of the pharmacokinetics of PEITC is important in order to understand the *in vivo* concentration-effect and concentration-toxicity relationships and to design dosing regimens. Furthermore, the oral bioavailability of PEITC has not been determined, despite the facts that PEITC is widely present in the human diet and has often been dosed orally in most *in vivo* studies to investigate its chemopreventive activity.

Our laboratory has demonstrated that certain dietary ITCs inhibit the P-glycoprotein- (P-gp-) and multidrug resistance-associated protein 1- (MRP1-) mediated efflux of daunomycin and vinblastine in multidrug resistance cancer cells (19, 20). More recently, we found that ITCs are inhibitors of breast cancer resistance protein (BCRP), a newly discovered ATP-binding cassette transporter, and 10 or 30 μM concentrations of ITCs could inhibit BCRP significantly (21). Due to the high expression of BCRP, MRP2 and P-gp in the human intestine as well as in human liver (22,23), ITCs may play a role in food-drug interactions by affecting the absorption and elimination of substrates for these transporters. Therefore, knowing the *in vivo* concentration ranges, oral bioavailability and pharmacokinetics of ITCs are important in our understanding of the effects of ITCs in food-drug interactions and multidrug resistance.

The present study examines the dose-dependent pharmacokinetics and oral bioavailability of PEITC in the rat animal model. This type of information cannot be obtained in humans since it requires intravenous (i.v.) administration of PEITC. The rat was used as the animal model because the metabolic disposition of PEITC is similar in rats and humans. We first examined the pH-dependent stability and serum protein binding of PEITC. Information regarding the stability of PEITC is essential

for establishing sample handling procedures for the pharmacokinetic study. We then determined the dose-dependent pharmacokinetics of PEITC in rats following oral and i.v. administration. Finally, a compartmental model was established to characterize the plasma concentration-time profiles obtained.

MATERIALS AND METHODS

Materials

PEITC, hydroxypropyl- β -cyclodextrin and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). 1,1,2,2- $^2\text{H}_4$ -PEITC was synthesized and characterized in our laboratory as described previously (24). ^{14}C -PEITC was synthesized in our laboratory according to Conaway et al., 1999 (17), with specific activity of 0.04994 mCi/mg and concentration of 1 mCi/ml. All the solvents used for HPLC and LC/MS/MS analysis were HPLC grade and were purchased from Fisher Scientific (Springfield, NJ).

Stability of PEITC

A universal buffer of citrate-phosphate-borate/HCl (Teorell & Stenhagen) was used to study the stability of PEITC. The buffer solutions, with pH of 3.0, 5.0, 7.4, 8.4, 9.3 and 10.1, were spiked with a 0.1mM PEITC (in acetonitrile). All samples were left at room temperature (RT) and analyzed at various times throughout a 120-hour period using HPLC assay (25). The effect of storage temperature on the stability of PEITC was also determined. The degradation rate constant (k_d) was calculated from the slope of the degradation plot (log (% remaining) vs. time) and the degradation half-life ($t_{1/2,d}$) was calculated using the equation $t_{1/2,d} = \ln 2 / k_d$.

Serum protein binding of PEITC

Protein binding measurements were determined by equilibrium dialysis using Spectra/Pro Dialysis Tubing (Rancho Dominguez, CA) with a 12,000 to 14,000 molecular weight cut-off at 37°C. Equal volumes (0.36 ml) of 0.13 M phosphate buffer (pH 7.4) and rat (Harlan Sprague Dawley) serum were added to each side of the dialysis chamber. ^{14}C -PEITC was added to the rat serum to yield concentrations

ranging from 10 to 1,000 μM . The samples were measured by liquid scintillation counting (1900 CA, Tri-Carb liquid scintillation analyzer, Packard Instruments Co., Meriden, CT). The unbound fraction (f_u) of PEITC was calculated as the ratio of free concentration and the total concentration.

Pharmacokinetic study of PEITC

Male Sprague-Dawley rats weighing 230 to 260 g were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and acclimated to their surroundings for about 1 week with food and water provided *ad libitum*. The research protocol was approved by the Institutional Animal Care and Use Committee at the University at Buffalo. Cannulas were implanted in the jugular vein of the rats two days prior to the study day. Rats were fasted overnight and each dosing group consisted of 3 or 4 animals. PEITC was prepared in 15% hydroxy-propyl- β -cyclodextrin under vigorous stirring at 50 °C followed by sonication for 5 minutes. Intravenous doses were administered via the jugular vein cannula at doses of 2, 10, 100 or 400 $\mu\text{mol/kg}$ and oral doses were given by gavage at doses of 10 and 100 $\mu\text{mol/kg}$. Water was provided at all times during the study and food was not available until 12 h after beginning the study. Blood samples (150 μl per sample) were collected into heparinized polypropylene tubes via the jugular vein cannula at 0, 5, 15, 30 min and 1, 2, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h following PEITC administration. After centrifugation at 1000 g for 6 min, the plasma was transferred into polyethylene tubes and kept frozen at -80°C until analysis.

LC/MS/MS Analysis

The sample extraction and LC/MS/MS analysis of PEITC in rat plasma were performed according to the previously described procedure with minor modifications

(24). Briefly, an aliquot of 100 μ l of original or diluted rat plasma was transferred into a 3 ml glass tube and 1,1,2,2- $^2\text{H}_4$ -PEITC was added as the internal standard. Two extractions were performed using 200 μ l of n-hexane. The hexane extracts were combined and 1 ml of ammonia (2M in 2-propanol) was added to derivatize PEITC to phenethylthiourea. The mixture was then dried under a N_2 stream and reconstituted with acetonitrile/ H_2O (3:2, v/v). The reconstituted sample was analyzed by LC/MS/MS using a PE SCIEX API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a heated nebulizer interface, a series 2000 Perkin-Elmer pump, and a series 2000 Perkin-Elmer autosampler (Shelton, CT). HPLC separation was performed on a C_{18} (particle size 5 μm ; 150 \times 4.6mm) column (Alltech, Deerfield, IL) with a mobile phase consisted of acetonitrile/5mM formic acid (60:40, v/v). The mass spectrometer was operated in a positive ionization mode and multiple reaction monitoring (MRM) of MS/MS was used for specific detection of the derivatives of PEITC and the internal standard. The assay recovery of PEITC in rat plasma ranged from 89.5 ± 3.1 to 98.3 ± 6.2 % for the concentrations from 50 to 1500 nM. The quantitation limit was 4 nM and the intra- and inter-day coefficients of variation were less than 5% and 10% respectively.

Pharmacokinetic Analysis

The plasma concentration data was analyzed by non-compartmental and compartmental analyses using WinNonlin Professional Edition Version 2.1 (Pharsight, Mountainview, CA) and ADAPT II (Biomedical Simulations Resource, University of Southern California, Los Angeles, CA), respectively. For non-compartmental analysis, the area under the plasma concentration vs. time curve (AUC) was determined using the log-linear trapezoidal rule with extrapolation to infinite time.

The elimination half-life ($t_{1/2}$) was estimated from the elimination rate constant (k) using the equation $t_{1/2} = \ln 2 / k$, where k was determined from the terminal slope of the plasma concentration vs. time curve. Clearance (Cl) was calculated by dividing the dose by AUC. The maximal plasma concentration (C_{\max}) and time to reach C_{\max} (t_{\max}) were determined directly from the plasma concentration-time curve. Volume of distribution (V_{ss}) was determined by $V_{ss} = Cl \cdot (AUMC/AUC)$, where AUMC is the area under the first moment curve ($C \cdot t$ vs. t curve). Oral bioavailability (F) was determined by the ratio of the dose-normalized AUCs following oral and i.v. administration.

For the compartmental analysis, all i.v. and oral plasma concentration data were fitted simultaneously. The initial estimates for model parameters were estimated based on the values obtained from non-compartmental analysis. The appropriate model was selected using the criteria of goodness-of-fit including visual inspection, Akaike's Information Criterion, Schwarz Criterion and correlation coefficient (r^2). Primary parameters derived from the model included first-order absorption rate constant (k_a), volume of distribution in central compartment (V_1), Michaelis-Menten constant and maximum velocity characterizing elimination ($V_{\max 1}$ and K_{m1}), volume of distribution in peripheral compartments (V_2 and V_3), peripheral clearance (Cl_{d2} and Cl_{d3}), and Michaelis-Menten constant and maximum velocity characterizing peripheral distribution ($V_{\max 3}$ and K_{m3}).

Statistical analysis

Statistical analysis was conducted using a one-way ANOVA followed by Dunnett's test or Bonferroni's test, or using a student's t test.

RESULTS

Stability of PEITC

The stability data of PEITC in buffers at various pH values are summarized in Table I.

PEITC was found to degrade in the buffer solutions by first order kinetics (Fig 2).

PEITC, when added to a buffer of pH 7.4, was more stable than that at pH 9.3 or pH 10.1, with degradation half-lives ($t_{1/2,d}$) of 56.1, 33.8 and 15.3 h, respectively ($p < 0.001$). On the other hand, PEITC had a longer $t_{1/2,d}$ at pH 3 (68.2 h) than at pH 7.4 ($p < 0.05$). At pH 7.4, when stored at a refrigerated temperature (4 °C), the $t_{1/2,d}$ of PEITC significantly increased to 108 h ($p < 0.001$) (Table I, Fig 2).

Protein Binding of PEITC

The optimal equilibration time for PEITC in the equilibrium dialysis experiments was 2 hours. As can be seen in Table II, PEITC was highly protein bound and the unbound fraction of PEITC in rat serum ranged from 0.0147 ± 0.0005 to 0.0227 ± 0.0005 over the concentration range of 10-1,000 μM . The average serum unbound fraction value of PEITC was 0.019. Ultrafiltration studies could not be performed since the binding of PEITC to Centrifree membranes was high; about 86 % of the added PEITC was bound to the membrane.

Pharmacokinetics Studies with Intravenous PEITC Administration

The plasma concentration vs. time profiles of PEITC following its i.v. administration are shown in Fig 3. At the lowest dose level of 2 $\mu\text{mol/kg}$, AUC, Cl, V_{ss} and $t_{1/2}$ values were $2.96 \pm 0.78 \mu\text{M}\cdot\text{h}$, $0.70 \pm 0.17 \text{ L/h/kg}$, $1.94 \pm 0.42 \text{ L/kg}$ and 3.52 ± 0.35 hours, respectively (Table III). When the dose was increased to 10, 100 and 400 $\mu\text{mol/kg}$, the AUC increased in a greater than proportional manner to 17.3 ± 9.3 , 322

± 149 and $807 \pm 66.9 \mu\text{M}\cdot\text{h}$, respectively (Table III). The $t_{1/2}$ at the medium dose level ($100 \mu\text{mol/kg}$) was significant longer than that at the dose of $2 \mu\text{mol/kg}$ ($p < 0.05$), and the $t_{1/2}$ at the highest dose level ($400 \mu\text{mol/kg}$) was significantly longer than that at doses of 2 ($p < 0.001$) or $10 \mu\text{mol/kg}$ ($p < 0.01$) (Table III). The $t_{1/2}$ obtained at the $2 \mu\text{mol/kg}$ dose is shown in parenthesis in Table III since the value may be underestimated due to the fact that samples at time points later than 24 h had concentrations lower than the quantitation limit of the assay. Nonetheless, the slower elimination at higher doses suggested the involvement of non-linear processes. Cl values were 0.70 ± 0.17 and $0.68 \pm 0.29 \text{ L}\cdot\text{h}^{-1}\cdot\text{kg}^{-1}$ at doses of 2 and $10 \mu\text{mol/kg}$, and decreased to $0.36 \pm 0.18 \text{ L}\cdot\text{h}^{-1}\cdot\text{kg}^{-1}$ at the medium dose ($100 \mu\text{mol/kg}$) and $0.50 \pm 0.04 \text{ L}\cdot\text{h}^{-1}\cdot\text{kg}^{-1}$ at the high dose ($400 \mu\text{mol/kg}$) (Table III), although these changes were not statistically significant. V_{ss} remained unchanged at doses of 2 , 10 and $100 \mu\text{mol/kg}$ and was 1.94 ± 0.42 , 3.27 ± 2.06 , $2.66 \pm 1.22 \text{ L/kg}$, respectively (Table III), but V_{ss} at the $400 \mu\text{mol/kg}$ dose ($5.72 \pm 1.10 \text{ L/kg}$) was significantly higher than that at 2 or $100 \mu\text{mol/kg}$ dose levels ($p < 0.05$), suggesting non-linearity in drug distribution following the highest dose.

To further examine the nonlinear nature of PEITC pharmacokinetics, the ratio values of AUC and dose were calculated and the plasma concentrations were normalized by dose. For linear pharmacokinetics, the AUC/dose ratio should remain constant due to a proportional increase in AUC with dose and the dose-normalized plasma concentration profiles of different doses should be superimposable. In this study, the ratios of AUC/dose varied, although not differed significantly; and the values were 1.48 ± 0.39 , 1.73 ± 0.93 , 3.22 ± 1.49 , and $2.02 \pm 0.17 \text{ kg}\cdot\text{h}\cdot\text{L}^{-1}$ following the doses of

2, 10, 100 and 400 $\mu\text{mol/kg}$, respectively (Table III). After normalization by dose, the plasma concentration curves did not superimpose at the four different dose levels (Fig 4), suggesting again that the pharmacokinetics of PEITC are not linear.

Pharmacokinetic Studies with Oral PEITC Administration

The PEITC plasma concentration-time profiles after oral administration are shown in Fig 5. PEITC was rapidly absorbed and the plasma concentrations peaked at 0.44 ± 0.10 and 2.0 ± 1.0 h after doses of 10 and 100 $\mu\text{mol/kg}$, respectively (Table IV). C_{max} was 9.2 ± 0.6 and 42.1 ± 11.4 μM after doses of 10 and 100 $\mu\text{mol/kg}$, respectively. The increase of C_{max} was not proportional to the increase of dose. F decreased as the dose increased, and was 114 and 93% at doses of 10 and 100 $\mu\text{mol/kg}$. The AUC values obtained after i.v. and oral administration of 10 and 100 $\mu\text{mol/kg}$ doses were not significantly different, suggesting that the oral bioavailability after these two doses is close to 1.

Compartmental Modeling of PEITC Plasma Concentration-Time Data

Different linear and non-linear kinetic models were used to simultaneously fit the plasma concentration data following i.v. and oral administration, including a two-compartment model, two-compartment model with Michaelis-Menten elimination, two-compartment model with Michaelis-Menten elimination and distribution, three-compartment model, three-compartment model with Michaelis-Menten elimination, three-compartment model with Michaelis-Menten elimination and distribution. Our results showed that a three-compartment model with Michaelis-Menten functions for both the central elimination and for one distribution term provided the best fitting (Fig. 6A and 6B). The r^2 values were 0.863, 0.625, 0.989, 0.997, 0.943 and 0.919 for the

curves following oral doses of 10 and 100 and i.v. doses of 2, 10, 100 and 400 $\mu\text{mol/kg}$, respectively. The fitted parameters by ADAPT II using this model are summarized in Table V.

The differential equations derived for the model (Fig 6A) were as follows:

$$\frac{dA}{dt} = -k_a \cdot X_a (X_{a,0} = \text{Dose}) \quad (1)$$

$$\frac{dC_1}{dt} = \frac{k_a \cdot X_a}{V_1} + \frac{Cl_{d2} \cdot C_2}{V_1} + \frac{V_{\max 3}}{K_{m3} + C_3} \cdot \frac{C_3}{V_1} - \frac{(Cl_{d2} + Cl_{d3}) \cdot C_1}{V_1} - \frac{V_{\max 1}}{K_{m1} + C_1} \cdot \frac{C_1}{V_1} (C_{1,0} = 0) \quad (2)$$

$$\frac{dC_1}{dt} = \frac{Cl_{d2} \cdot C_2}{V_1} + \frac{V_{\max 3}}{K_{m3} + C_3} \cdot \frac{C_3}{V_1} - \frac{(Cl_{d2} + Cl_{d3}) \cdot C_1}{V_1} - \frac{V_{\max 1}}{K_{m1} + C_1} \cdot \frac{C_1}{V_1} (C_{1,0} = \text{Dose}) \quad (3)$$

$$\frac{dC_2}{dt} = \frac{Cl_{d2} \cdot C_1}{V_2} - \frac{Cl_{d2} \cdot C_2}{V_2} (C_{2,0} = 0) \quad (4)$$

$$\frac{dC_3}{dt} = \frac{Cl_{d3} \cdot C_1}{V_3} - \frac{V_{\max 3}}{K_{m3} + C_3} \cdot \frac{C_3}{V_3} (C_{3,0} = 0) \quad (5)$$

The initial conditions for each equation are shown in parenthesis. Equation (1), (2), (4) and (5) were used to fit the oral data and equation (3), (4) and (5) were used to fit the i.v. data.

DISCUSSION

PEITC is widely present in cruciferous vegetables as its glucosinolate precursor, gluconasturtiin. An intake of 2 oz (56.8g) of watercress releases a minimum of ~12mg of PEITC (16), and the amount of gluconasturtiin in Chinese cabbage is between 2 and 26 mg/100g fresh weight (26). Hence, human consumption of PEITC through the diet is significant. More importantly, the compound possesses potent anticarcinogenic effects, and as such, PEITC is of extensive research and clinical interest. Recently, our laboratory found that PEITC could inhibit certain ATP-binding cassette transporters. However, many properties of this compound, namely its stability, protein binding and *in vivo* pharmacokinetics are largely unknown.

In the present work, stability of PEITC was studied in universal buffers of citrate-phosphate-borate/HCl at pH values of 3.0, 5.0, 7.4, 8.4, 9.3 and 10.1. All samples were studied following incubation at RT and at refrigerated temperatures. The stability of PEITC was both pH- and temperature-dependent. PEITC degraded in a first-order manner and the $t_{1/2,d}$ at pH 7.4 at RT was 56 h. Within the investigated pH range, PEITC is most stable at pH 3 and least stable at pH 10.1, with a $t_{1/2,d}$ decreasing from 68.2 to 15.3 h. The degradation of PEITC at pH 7.4 was significantly decreased at refrigerated temperatures, with a $t_{1/2,d}$ of 108 h at 4 °C. In general, our results showed that PEITC is stable in aqueous buffers at biological pH, and that stability increased if samples were maintained at refrigerated temperatures. Our results contrasted with the report by Negrusz et al., who indicated that PEITC is not stable at low pH in aqueous media and degrades to phenethylamine (27). Nonetheless, PEITC was found to be stable during sample collection and handling, provided that samples

are stored under refrigerated conditions. In our pharmacokinetic study, all samples were handled immediately after collection and stored at -80°C until analysis.

In this study, we used rat serum to examine the concentration-dependent binding of PEITC. Over the concentration range of 10-1000 μM , the binding of PEITC to serum proteins was not dependent on the compound concentration, with an average free fraction of 0.019. These results suggested that PEITC is extensively bound to protein in rat serum, likely due to the lipophilic nature of PEITC ($\log P$ of 3.47).

An examination of the dose-dependent pharmacokinetics of PEITC demonstrated that the clearance of PEITC decreased with increasing doses, suggesting that PEITC is eliminated in a capacity-limited manner. At doses of 2, 10, 100 and 400 $\mu\text{mol/kg}$ of PEITC, $t_{1/2}$ values were 3.52 ± 0.35 , 6.92 ± 3.73 , 9.19 ± 0.83 and 13.1 ± 2.0 hours, respectively. The $t_{1/2}$ values are smaller than the $t_{1/2}$ of 20.5 hours reported in rats after the administration of a 50 $\mu\text{mol/kg}$ oral dose of ^{14}C -PEITC (17). This longer $t_{1/2}$ of ^{14}C -PEITC may be due to the slower clearance or larger volume of distribution of metabolites that contain the isotope. The major metabolic route of PEITC in rats is the mercapturic acid pathway, where PEITC undergoes glutathione conjugation followed by hydrolysis to the cysteine derivative and finally N-acetylation (10). At a 400 $\mu\text{mol/kg}$ dose of PEITC, the clearance of PEITC was significantly slower than those of doses of 2 and 10 $\mu\text{mol/kg}$, which could be due to saturation of glutathione S-transferase and/or any other enzymes involved in the mercapturic acid pathway.

The V_{ss} for PEITC ranged from 1.94 to 5.72 L/kg at doses of 2 to 400 $\mu\text{mol/kg}$. The high values of V_{ss} suggest that PEITC may be extensively bound to tissues. The

electrophilic carbon atom of the isothiocyanate group (—N=C=S) reacts rapidly with oxygen-, sulfur-, or nitrogen- centered nucleophiles (6). It is widely accepted that ITCs covalently bind to proteins through preferentially reacting with sulfhydryl groups of amino acid residues of some proteins. Our protein binding data also indicated that PEITC is highly protein bound. Therefore, PEITC may permeate into tissues and bind to tissue proteins extensively, resulting in low unbound tissue concentrations and a high volume of distribution. The V_{ss} value at 400 $\mu\text{mol/kg}$ was significantly higher than those at 100 and 2 $\mu\text{mol/kg}$. This nonlinearity could be due to saturable active efflux from the tissues to the plasma. The glutathione conjugate of PEITC (PEITC-SG) is believed to be a substrate for MRP1 (20,28). MRP1 relies on the energy from ATP hydrolysis to efflux various anticancer drugs and toxins, and is present ubiquitously in the human body and tumors including both solid tumors and hematological malignancies (29,30). At high doses, the metabolism of PEITC to PEITC-SG may be saturated or MRP1-mediated efflux may be inhibited due to depletion of intracellular glutathione (20), resulting in higher PEITC tissue concentrations and thus a higher apparent volume of distribution.

Following oral administration of PEITC, peak plasma concentrations occurred rapidly at 0.4 ± 0.1 and 2.0 ± 1.0 hours following doses of 10 and 100 $\mu\text{mol/kg}$ with a k_a of 1.8 h^{-1} . In our previous clinical study, PEITC has an apparent absorption rate constant (k_a) of 1.3 h^{-1} and t_{\max} of 2.6 hours in healthy subjects after consumption of 100 g watercress (24). The values are comparable to what we observed in rats. Taken together, PEITC exhibits rapid absorption and high availability. Conaway et al. reported that peak whole blood ^{14}C concentrations occurred at 2.9 hours in rats following a dose of 50 $\mu\text{mol/kg}$ ^{14}C -PEITC (17). The longer t_{\max} reported when

measuring the ^{14}C radiolabel may reflect the contribution of hydrophilic metabolites, formed from PEITC, to the total radioactivity measured. In that study, the mean whole blood ^{14}C C_{max} value was $18.77\ \mu\text{M}$ (17), which is within the range we observed for our 10 and $100\ \mu\text{mol/kg}$ doses (C_{max} values of 9.2 ± 0.6 and $42.1 \pm 11.4\ \mu\text{M}$, respectively).

Following doses of 10 and $100\ \mu\text{mol/kg}$, the F values of PEITC were 115% and 93%, respectively, and the AUCs were not significantly different for oral and i.v. administration, suggesting PEITC was almost completely absorbed after oral administration. As a small hydrophobic compound, PEITC permeates cells easily by passive diffusion; therefore, good intestinal absorption would be expected. In addition, high oral bioavailability suggests that first-pass metabolism may be negligible. Although PEITC can undergo glutathione conjugation, this may not be extensive in the intestine and liver; alternatively, or in addition, PEITC-SG may undergo hydrolysis, releasing PEITC that can diffuse out of the intestinal cells and hepatocytes.

The compartment model presented was chosen based on goodness of fit and for its physiological relevance. Compartment I represents the central compartment, where PEITC was eliminated in a capacity-limited manner, as a result of saturable metabolism. Compartment II represents the tissues into which PEITC diffuses freely and distributes in a linear pattern. Compartment III represents those tissues that express MRP1 or other forms of MRP, where there is active efflux of PEITC/PEITC-SG. PEITC enters cells by passive diffusion; therefore, Cl_{d3} , the peripheral clearance from compartment I to III is linear. Once PEITC enters cells, it may conjugate with

glutathione rapidly. It has been reported that the intracellular accumulation of ITC-SG is maximal after about 30 min in cultured cells (31), and the intracellular ITC-SG may be further metabolized, or hydrolyzed to liberate ITCs due to the reversible conjugation (32). Therefore, if not hydrolyzed back to PEITC, the formed PEITC-SG conjugate may be effluxed by MRP1 back to the central compartment. The fitted K_{m1} and K_{m3} were 109 and 1.9 μM , respectively. At low PEITC concentrations, the plasma concentration-time profile could be fit by a linear model since plasma concentrations were much lower than the two K_m values; at high concentrations, greater than the K_m , the non-linear nature became evident. Our analysis showed that following a 2 $\mu\text{mol/kg}$ dose, the plasma concentration of PEITC exhibited an apparent linear two-compartment model. When the dose was increased, however, a three-compartment model fit the plasma concentration data better. When the dose was increased 200-fold to 400 $\mu\text{mol/kg}$ and plasma samples were collected up to 72 hours, a three-compartment model with saturable efflux compartment best fit the plasma concentration data. Previously, the time course of whole blood radioactivity, following an oral dose of 50 $\mu\text{mol/kg}$ ^{14}C -PEITC to rats, has been reported to fit a two-compartment linear model (17). This difference, from the present investigation, may be due to the fact that the ^{14}C reflects the total of PEITC and its metabolites; the metabolite disposition may be different from that of the parent. Additionally, the sampling was sparse, especially during the distribution phases, compared with the present investigation.

In summary, PEITC degrades in aqueous buffers with a half-life of 56.1 hours at a pH 7.4 at RT and this degradation half-life increases to 108 hours when samples are stored at 4 °C. The protein binding of PEITC is high (98.1%) and is not

concentration-dependent. Oral bioavailability of PEITC in rats is high and was 115% and 93% following doses of 10 and 100 $\mu\text{mol/kg}$, respectively. The clearance following a 2 $\mu\text{mol/kg}$ dose of PEITC was $0.7 \pm 0.17 \text{ L/h/kg}$ and the mean value was decreased at higher doses. The V_{ss} following a 2 $\mu\text{mol/kg}$ dose of PEITC was $1.94 \pm 0.42 \text{ L/kg}$ but increased to 5.72 ± 1.10 following a dose of 400 $\mu\text{mol/kg}$. A three-compartment model with Michaelis-Menten elimination and distribution provided the best fit of the plasma concentration-time data following oral and i.v. administration in rats.

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TABLES

Table I. Stability of PEITC in universal buffers at different pH values and temperatures.

pH	k_d (h^{-1})	$t_{1/2,d}$ (h)	Temperature ($^{\circ}C$)
7.4	0.0125 ± 0.0013	56.1 ± 6.0	25
3.0	0.0104 ± 0.0015	$68.2 \pm 9.8^*$	25
5.0	0.0111 ± 0.0018	64.3 ± 10.6	25
8.4	0.0111 ± 0.000004	62.3 ± 0.02	25
9.3	0.0205 ± 0.0005	$33.8 \pm 0.8^{**}$	25
10.1	0.0462 ± 0.0067	$15.3 \pm 2.2^{**}$	25
7.4	0.0064 ± 0.0003	$108.1 \pm 4.3^{**}$	4

All experiments were performed at RT (25 $^{\circ}C$) or 4 $^{\circ}C$. The degradation half-life ($t_{1/2,d}$) was calculated from the slope of the PEITC degradation plot (log (% remaining) vs pH). One-way ANOVA followed by Dunnett's test was used to compare $t_{1/2,d}$ at different experimental conditions to $t_{1/2,d}$ at pH 7.4 under RT. Data is expressed as mean \pm SE, n = 6 (RT), n = 3 (4 $^{\circ}C$), * $p < 0.05$, ** $p < 0.001$.

Table II. Protein binding of ^{14}C -PEITC in rat serum.

PEITC Conc. (μM)	f_u (%)
10	1.47 ± 0.05
50	1.65 ± 0.01
100	1.69 ± 0.04
250	1.82 ± 0.08
500	2.02 ± 0.03
750	2.27 ± 0.21
1000	2.27 ± 0.05

Protein binding of ^{14}C -PEITC was determined using equilibrium dialysis at 37°C using Spectra/Pro dialysis tubing with a molecular weight cut-off of 12,000-14,000 MW; f_u represents the unbound fraction of PEITC and was calculated as the ratio of free concentration over the total concentration. Data is expressed as mean \pm SD, $n = 3$.

Table III. Pharmacokinetic parameters of PEITC after intravenous administration determined by non-compartmental analysis.

Dose ($\mu\text{mol/kg}$)	2	10	100	400
AUC ($\mu\text{M}\cdot\text{h}$)	2.96 ± 0.78	17.3 ± 9.3	322.1 ± 149.6	807.5 ± 66.9
$t_{1/2}$ (h)	(3.52 ± 0.35)	6.92 ± 3.73	$9.19 \pm 0.83^{**}$	$13.1 \pm 2.0^{***\Delta}$
Cl (L/h/kg)	0.70 ± 0.17	0.68 ± 0.29	0.36 ± 0.18	0.50 ± 0.04
V_{ss} (L/kg)	1.94 ± 0.42	3.27 ± 2.06	2.66 ± 1.22	$5.72 \pm 1.10^{**\S\S}$
AUC/Dose ($\text{kg}\cdot\text{h/L}$)	1.48 ± 0.39	1.73 ± 0.93	3.22 ± 1.49	2.02 ± 0.17

Noncompartmental analysis was performed by WinNonlin. Abbreviations for the parameters: AUC, area under the plasma concentration vs. time curve; $t_{1/2}$, elimination half-life; Cl, clearance; V_{ss} , volume of distribution. Statistics were conducted by ANOVA followed by Bonferroni's test, $n = 3$ or 4 ; $^{**}P < 0.05$ and $^{***}P < 0.001$ compared to the group administered with $2 \mu\text{mol/kg}$ PEITC; $^{\Delta}P < 0.01$ compared to the group administered with $10 \mu\text{mol/kg}$ PEITC; $^{\S\S}P < 0.05$ compared to the group administered with $100 \mu\text{mol/kg}$ PEITC.

Table IV. Pharmacokinetic parameters of PEITC after oral administration
determined by non-compartmental analysis

Dose ($\mu\text{mol/kg}$)	10	100
AUC ($\mu\text{M}\cdot\text{h}$)	19.89 ± 3.27	298.7 ± 139.4
t_{max} (h)	0.44 ± 0.10	2.0 ± 1.0
C_{max} (μM)	9.2 ± 0.6	42.1 ± 11.4
F (%)	115	93

Noncompartmental analysis was performed by WinNonlin. Abbreviations for the parameters: C_{max} , maximal plasma concentration; t_{max} , time to reach C_{max} ; F, bioavailability.

Table V. Estimated pharmacokinetic parameters of PEITC in rats after intravenous and oral administration determined by compartmental analysis

Parameter	Unit	Estimated value
k_a	h^{-1}	1.8
V_1	L/kg	0.70
V_{max1}	$\mu\text{mol/h/kg}$	52.9
K_{m1}	μM	109
Cl_{d2}	L/h/kg	0.69
V_2	L/kg	1.56
Cl_{d3}	L/h/kg	0.054
V_3	L/kg	11.6
V_{max3}	$\mu\text{mol/h/kg}$	1.35
K_{m3}	μM	1.9

LEGENDS FOR FIGURES

Fig 1. The chemical structure of PEITC

Fig 2. Stability of PEITC in universal buffers determined over 120 hours. PEITC in buffer with pH values of 3.0 (●), 5.0 (▼), 7.4 (○), 8.3 (▽), 9.3 (■) and 10.1 (□) at room temperature and pH of 7.4 (◆) at 4°C.

Fig 3. The plasma concentration profile of PEITC after intravenous administration. Rats were intravenously dosed with 2 (●), 10 (○), 100 (▲) or 400 $\mu\text{mol/kg}$ (Δ) of PEITC. Data are expressed as mean \pm SD, $n = 3$ or 4.

Fig 4. The dose-normalized plasma concentration of PEITC in rats after intravenous administration. Rats were intravenously dosed with 2 (●), 10 (○), 100 (▲) or 400 $\mu\text{mol/kg}$ (Δ) of PEITC. Data are expressed as mean \pm SD, $n = 3$ or 4.

Fig 5. The plasma concentration profile of PEITC after oral administration. Rats were intravenously dosed with 10(▼) and 100 $\mu\text{mol/kg}$ (▽) of PEITC. Data are expressed as mean \pm SD, $n = 3$ or 4.

Fig 6. The proposed compartmental model (A) and the observed and predicted plasma concentrations of PEITC (B) after intravenous and oral administration. X_a represent the drug amount at the absorption site; C_1 , C_2 and C_3 represent the plasma concentrations of PEITC in central (I) and two peripheral compartments (II and III), respectively; V_1 , V_2 and V_3 represent the volume of distribution of PEITC in central (I) and two peripheral compartments (II and III), respectively; k_a is the absorption rate constant; K_{m1} and $V_{\max1}$ are the Michaelis-Menten parameters to characterize central clearance from central compartment; Cl_{d2} represents peripheral clearance between peripheral compartment II and central compartment. Cl_{d3} represents peripheral clearance from central compartment to peripheral compartment III; K_{m3} and $V_{\max3}$ are the Michaelis-Menten parameters to characterize clearance from peripheral

compartment III to central compartment. Rats were dosed with 2 (●), 10 (○), 100 (▲) or 400 $\mu\text{mol/kg}$ (\triangle) of PEITC intravenously, or (\blacktriangledown) and 100 $\mu\text{mol/kg}$ (∇) orally.

Data are expressed as mean \pm SD, $n = 3$ or 4. All the data were fitted simultaneously by ADAPT II and the lines represent predicted plasma PEITC concentrations using the proposed compartmental model.

Fig 1.

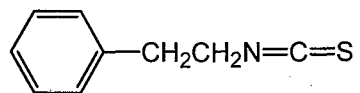


Fig 2.

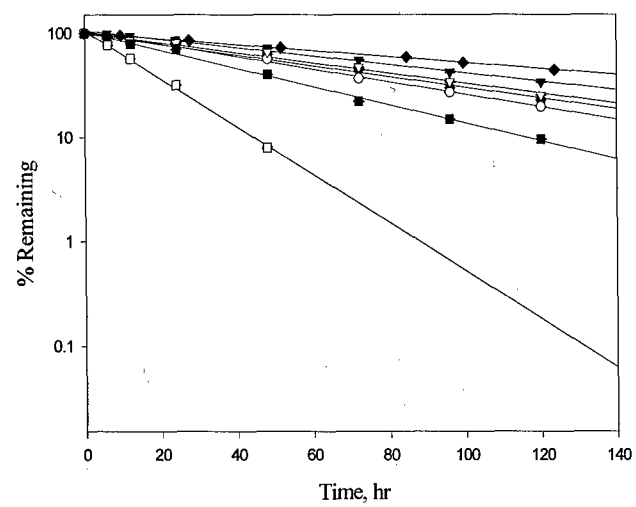


Fig 3.

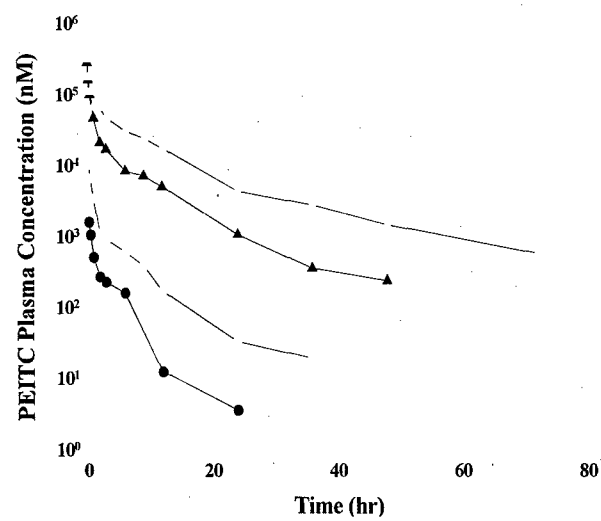


Fig 4.

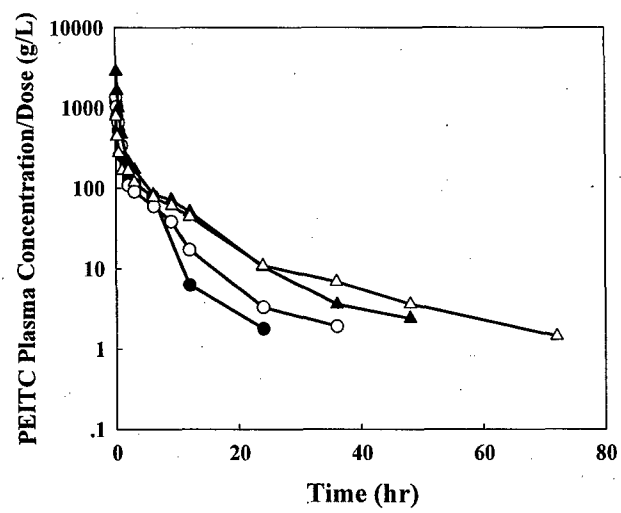


Fig. 5

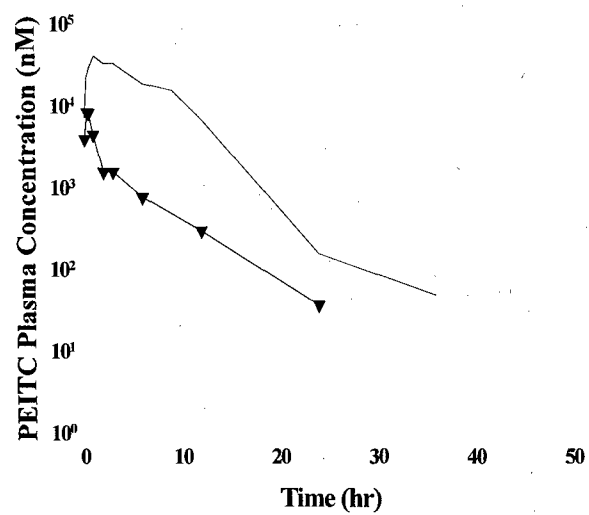
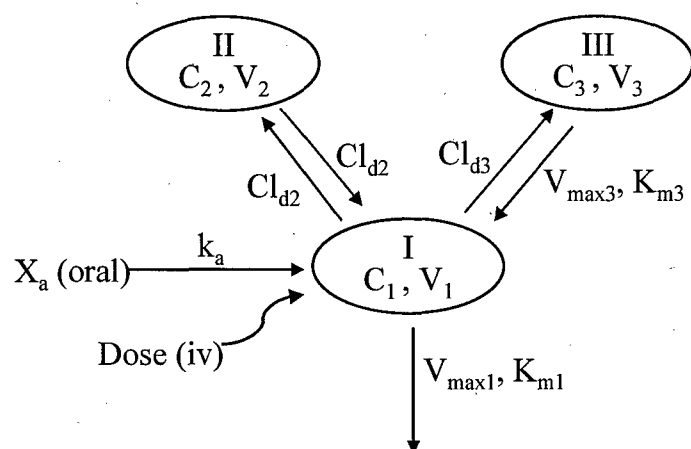


Fig. 6.

A



B

